대한생리학회지 : 제 17권 제 2 호

Effect of Vanadate on PAH Transport and Na-K-ATPase Activity in Rabbit Renal Cortex

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=국문요약=

가토 신피질에서 PAH 이동과 Na-K-ATPase 활성에 미치는 Vanadate의 영향

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Vanadate가 가토 신피질절편에서 PAH이동과 Na-K-ATPase 활성에 미치는 효과를 관찰한 결과 다음과 같은 결론을 얻었다.

1) Vanadate는 Na-K-ATPase 활성을 농도에 따라 억제하였으며 7.94×10^{-7}M에서 이 효소의 활성이 50% 억제되었다.

2) Vanadate는 PAH의 능동적이동을 농도에 따라 억제하였으며 50%억제농도는 대략 10^{-4}M이었고, 수동적이동에는 영향을 미치지 못하였다. 조직내 Na과 K의 양도 vanadate가 PAH이동을 억제하는 농도 범위에서 같이 변화하였고 산소소모량은 10^{-4}M까지는 약간 감소하였으나 10^{-3}M에서는 오히려 증가하였다.

3) 30분간 preincubation한 후에도 15분까지의 PAH이동은 30분 이후에 비해 vanadate에 의해 적게 억제되었다.

4) 10^{-4}M vanadate와 10^{-4}M ouabain은 가역적으로 PAH 이동을 억제하였으며 10^{-3}M vanadate는 비가역적으로 억제하였고 장시간 세척후에도 거의 같은 정도의 억제양상을 나타내었다.

5) Vanadate에 의한 PAH이동의 억제정도는 incubation용액내 Na^+의 감소, K^+의 증가에 의하여 증가하였고 Ca^{2+}의 농도 변화에 의해서는 영향을 받지 않았다.

6) Vanadate가 존재치 않을 때 Tris완충용액 사용시는 pH 8.2까지 PAH축적정도가 증가하였고 phosphate완충용액 사용시는 pH 7.4에서 최대축적치를 보였다. pH가 증가함에 따라 억제정도는 증가하였으며 같은 pH에서도 완충용액의 종류에 따라 vanadate에 의한 억제정도가 달랐다.

7) Vanadate와 ouabain은 PAH이동과 Na-K-ATPase 활성에 부가적 억제작용을 나타내었다.

이상의 결과로 vanadate는 가토신장의 세포내부에서 Na-K-ATPase를 가역적으로 억제함으로써 PAH의 이동을 억제하는 것으로 생각되며 PAH의 이동은 Na-K-ATPase 활성과 기능적으로 밀접히 연결되어 있는 것으로 생각된다.

INTRODUCTION

Vanadium (V, atomic wt.)[50,94] is a member of the Vb group of elements in the periodic table and shares several features in common with elements in the Va group (nitrogen, phosphorus, arsenic). Vanadium occurs naturally in the earth's crust principally in the trivalent state as relatively insoluble salts, the average concentration being about 150 mg/kg[61]. The element is found in a variety of plants

and animals, many of which are ingested by human beings[78]. It is probably an essential trace element for animals[41,72].

In solution vanadium forms oxyanions that resemble the more familiar phosphate compounds. Vanadate exists in different forms depending on the pH (VO_4^{3-}, HVO_4^{2-}, $H_2VO_4^-$). In biological fluids oxyvanadium exists principally in two redox states. Vanadium(+5) (vanadate, HVO_4^{2-}) is formed spontaneously in atmospheric oxygen from vanadium(+4) (vanadyl, VO^{2+})[5]. There are evidences which indicate that within cells vanadium is principally in the 4+ redox state, whereas in extracellular fluids vanadium is principally in the 5+ redox state[13,29,67]. Oxyvanadium compounds are absorbed from intestine and eliminated principally by the kidneys[42,81]. Most mammalian tissues contain traces of vanadium, the kidney containing the highest concentration, principally in the cortex[1,42,63,81,85]. Vanadium is also found in substantial amounts in liver and bone, the quantities being related to the size of these tissue reservoirs.

In 1975 Charney et al.[17] reported that a certain brand of ATP used in the assay of renal Na-K-ATPase contained "unwanted" inhibitor. Beauge and Glynn[5] and Hudgins and Bond[43] reported that a commercial brand of ATP contained a "modifier" of the Na-K-ATPase that caused potassium at concentrations above 5 mM to inhibit rather than to activate the enzyme. Cantley et al.[15] subjected the commercial ATP to electron probe and electron spin resonance analysis and discovered a strong vanadium signal and they showed that vanadate is more potent inhibitor of Na-K-ATPase than ouabain under optimal conditions. From that point on, interest in vanadate has been spurred by the fact that it was present in ATP extracted from horse muscle, but was not present in synthetic ATP. Since then, its biological effects has been extensively studied. The main physiological effects of vanadate are diuresis, natriuresis[1,24,90], the increase in the force of the contraction of the heart[40,80], and the contraction of the smooth muscle[44,69,84].

The existence of a renal seretory system for organic anion in mammals has been suspected since it was first observed that more phenol red appears in the urine of dogs than can be accounted for by filtration alone[53]. Para-aminohippurate(PAH) replaced phenol red as the model compound in the study of renal organic anion transport because it had the advantage of having low binding capacity to plasma proteins, of being completely removed from the plasma in a single pass through the kidney, and of negligible metabolism and convenient calorimetric assay[76]. Cross and Taggart[22] found that thin slices of the rabbit renal cortex can accumulate PAH to concentrations many times that of the bathing medium. The rabbit renal cortex consists predominantly of proximal tubules[79] and any fluid initially present in the lumens will be rapidly reabsorbed and then collapsed the lumen[8]. Therefore, cortical slice accumulation should represent transport across the basolateral membrane and accumulation within the proximal tubule cells. This was subsequently confirmed by direct visualization using transported dyes[33] and autoradiography using radiolabeled PAH[88]. In vivo confirmation of a proximal tubular site for PAH secretion has come from stop flow experiments[52] and micropuncture studies[21,82].

It has been known that PAH transport is dependent on both Na^+ and K^+ in the medium [18,35,36,65,66] and tissue metabolism[22,56], but the primary energy source of PAH transport is

not clear. Under anaerobic conditions, Podevin et al.[66] observed transient accumulation of PAH in the presence of Na^+-gradient between extracellular and intracellular side. Since glycolytic processes are not sufficient to drive PAH or Na^+ transport by proximal tubule cells, it was concluded that transient accumulation of PAH under anaerobic conditions probably occurs by co-transport with Na^+. It was further suggested by these authors that active transport of PAH under aerobic conditions is solely dependent on the thermodynamic energy of the Na^+-gradient. However, Gerencser and Hong[35] provided evidence suggesting that the accumulation of PAH under aerobic conditions is critically dependent on a sufficiently high intracellular concentration of Na^+, rather than on medium Na^+. They proposed that PAH transport is linked in a direct manner to the function of Na-K-ATPase. Maxild et al.[55] reported that the PAH uptake process can be divided into rapid equilibrating component and a slowly equilibrating component, which makes the largest contribution to the final accumulation of PAH. They insisted that the rapid uptake of PAH be dependent on oxidative metabolism and independent on Na^+-gradient, but the slow uptake process is dependent on Na^+. Recently, some investigators[28,73,75] reported that vanadate inhibits PAH transport in the renal cortex by the inhibition of Na-K-ATPase.

At first vanadate was considered to be a specific inhibitor of Na-K-ATPase and an integral part of a cellular control mechanism for the sodium pump in vivo[14]. However, recent studies showed that vanadate has the effects on several other enzymes. Vanadate inhibits Ca^{2+}-ATPase[68,87,91], alkaline phosphatase from microorganisms[50], actomyosin ATPase[38], dynein ATPase[37], and glucose-6-phosphatase[74]. Vanadate stimulates adenylate cyclase[71], although it blocks the water and sodium reabsorption to vasopressin in the isolated toad bladder[26]. Also, some actions of vanadate — vasoconstriction[69] and contraction of intestinal smooth muscle[44,84], inhibition of sodium transport and of sodium-independent anion transport in turtle bladder[29], the inhibition of hepatic bile production[83], and the secretion of renin from kidney[19,49] — cannot be explained through the inhibition of Na-K-ATPase because of no effect of vanadate on the intracellular electrolyte concentration and the flux of radioactive potassium. Therefore, it is possible that vanadate inhibits PAH transport through some mechanisms different from the inhibition of Na-K-ATPase in view of the diverse effects of vanadate on biological functions and the absence of ability to inhibit Na-K-ATPase in several intact tissues[7,47]. The objectives of the present experiment are to examine the effect of vanadate on the accumulation of PAH in rabbit kidney cortical slices and elucidate its action mechanism.

MATERIALS AND METHODS

Slice uptake

Fresh kidneys were obtained from rabbits weighing approximately 2 kg. After the animals had been put to death by the injection of air through the auricular vien, the kidney was promptly removed and placed in an ice cold saline solution (130 mM NaCl and 20 mM KCl). To remove the blood from the kidneys, they were perfused through the renal arteries with ice cold saline. Thin slices (0.3~0.4 mm) of renal cortex were then prepared using a Stadie-Riggs microtome and these were stored in ice cold saline prior to incubation.

The degree to which renal cortical tissue

could accumulate PAH was assessed by incubating cortical slices in a modified Cross-Taggart incubation medium whose composition was 100 mM NaCl, 40 mM KCl, 1.5 mM $CaCl_2$, 10 mM Na acetate, and 10 mM Tris at pH 7.8. 74 μM PAH and appropriate concentrations of vanadate were contained. The incubation procedure consisted of placing two or three renal cortical slices (approximately 200 mg) and 10 ml of incubation medium into 20 ml flasks and incubaing them in the presence of 100% O_2 at 25°C. After the tissue had been incubated for 40 min, they were removed and blotted, quickly and gently, on the gauze. Until the tissues could be weighed and homogenized, they were stored in a small flask to minimize evaporative weight loss by the tissues. The tissues were weighed using a top loading balance (Mettler PC-440). The distribution of PAH was determined by assaying the tissue and incubation medium after incubation. Tissue samples were homogenized in 4 ml of distilled water and deproteinized by adding 2 ml of 1 N NaOH and 6 ml of $CdSO_4$ in H_2SO_4. The samples were centrifued. Supernatant was used for measuring the amount of PAH. The incubation medium was treated in a similar manner. The determination of PAH was made by using the method of Smith et al.[76] Slice uptake rate is expressed as the slice-to-medium concentration ratio (S/M).

Washout experiment

Renal cortical slices were preincubated in medium containing various concentrations of vanadate without PAH for 40 min, after which they were blotted and transferred to fresh incubation medium. One half of slice were rinsed for 5 min in vanadate-free incubation medium and the other half in a medium containing the same concentration of vanadate as preincubation medium. The slices were removed from this intermediate wash, blotted and transferred to another fresh incubation medium of the same composition except the one containing 74 μM PAH as intermediate wash for additional 40 min. To obtain the control data, slices were treated as the same method in vanadate-free incubation medium. Concentrations of PAH in the slices and medium were determined as described above.

Passive uptake

Passive uptake of PAH by the tissue was measured by incubating the slices for 40 min in a medium which contained, in addition to the usual constituents, 600 μM probenecid with an N_2 gas phase.

Tissue electrolytes

After incubation period slices were blotted, weighed, and digested in 1 ml of concentrated HNO_3 for 24 hrs. After centrifugation, supernatant was used for measuring Na and K concentrations. Na and K concentrations in the samples were determined by flame photometry using lithium as an an internal standard. The flame photometer that was (Beckman Klina flame photometer) required the condition that the samples be diluted 1 : 200 in a 15 mM lithium chloride solution.

Oxygen consumption

The oxygen consumption of renal cortical slices was assessed by measuring the change in O_2 saturation of 3 ml of well stirred incubation medium containing approximately 50 mg of tissue. This was accomplished by using an oxygen monitor with Clark-type oxygen electrodes (model 53, Yellow Springs Instrument Co.). The datas were given as a initial rate of oxygen consumption computed from

the decrease in oxygen saturation of the bathing solution over a 15 min incubation at 25°C. The change in saturation was converted to μl of O_2 consumed per mg of tissue per hr using a solubility of O_2 in normal Ringers of 0.0375 μl O_2. ml^{-1} $torr^{-1}$ at 25°C (international critical tables, 1928).

Na-K-ATPase activity

The Na-K-ATPase activity was determined in the homogenate of rabbit kidney cortex. Rabbit was killed and the kidneys were removed and decapsulated. After blood was removed, the cortex was then collected by the sharp dissection. The pooled zones were suspended in a medium containing 250 mM sucrose, 5 mM EDTA, and 30 mM imidazole at pH 7.4 (g wet weight of tissue/10 ml), and homogenized in a Teflon-glass, motor-driven, Potter-Elvejem homogenizer (10 strokes). Aliquots of this crude homogenates were filtered through gauze and stored at 0~4°C and then analyzed for enzyme activity.

The Na-K-ATPase activity of the homogenate was determined by measuring the inorganic phosphate (Pi) released by ATP hydrolysis during incubation of homogenate within appropriate medium containing Na_2-ATP (Sigma) as the substrate. The total ATPase activity was determined in the presence of 50 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM ATP and 100 mM imidazole at pH 7.5. The Mg-ATPase activity was determined in the absence of K^+ with 0.1 mM ouabain present. The difference between the total and the Mg-ATPase activities was taken as a measure of the Na-K-ATPase activity. After preincubation at 37°C in the presence of 2 mM ATP, the reaction was initiated with the addition of 10 mM KCl. At the end of 10 min incubation, the reaction was terminated by the addition of ice cold 11.67 % perchloric acid. The mixture was then centrifuged, and the supernatant from these tubes was then assayed for Pi by the method of Fiske and SubbaRow[32]. The protein concentration was determined by Lowry et al.[51] with using bovine serum albumin as the standard.

Statistical analysis

The datas were reported as the mean value ±the standard error of the mean (SEM). The datas were evaluated for significance using Student's t-test. A probability level of 0.05 was used to establish significance.

Special reagents

Sodium orthovanadate (Fisher Scientific Co.)
Ouabain octahydrate (Sigma Co.)
Na_2-ATP (vanadate-free) (Sigma Co.)
p-Aminohippurate (Sigma Co.)

RESULTS

1) Effect of vanadate on Na-K-ATPase activity of rabbit renal cortex

The Na-K-ATPase activity by renal cortical homogenate was measured in the presence of varying concentrations of vanadate between 10^{-8} and 10^{-4}M. In vanadate-free medium, Na-K-ATPase activity was 2.77±0.20 μmol Pi/mg protein/10 min. In the presence of vanadate Na-K-ATPase activity was markedly inhibited in a dose-dependent manner (Fig. 1). The vanadate concentration at which activity was inhibited by 50% (ID_{50}) was 7.94×10^{-7}M. This result was consistent with that of Nieder et al. (62), but not with that of Cantley et al.[15] and Grantham and Glynn (39). This is most likely attributed to assay conditions. 10^{-4}M vanadate inhibited Na-K-ATPase activity completely.

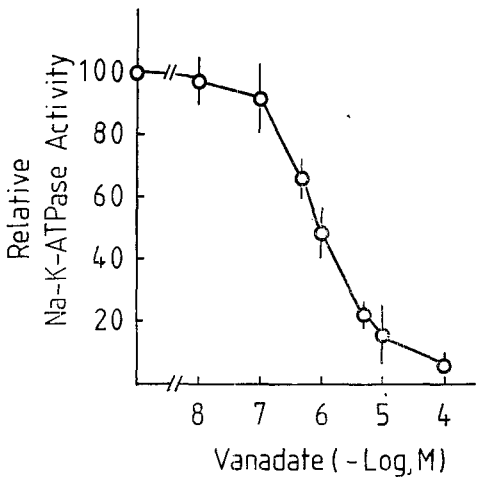

Fig. 1. Effect of vanadate on Na-K-ATPase activity. These values represent mean±SEM of 5 experiments.

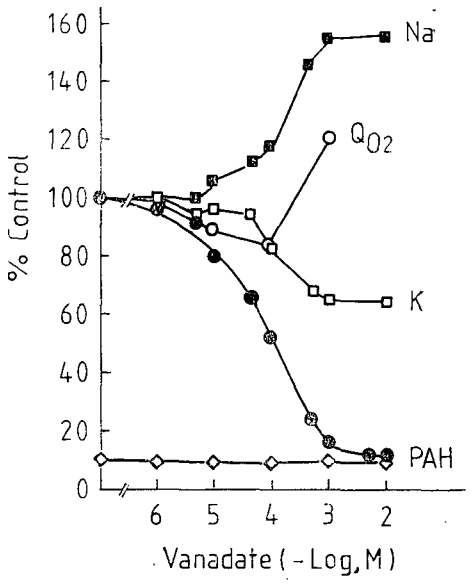

Fig. 2. Effects of vanadate on PAH uptake, tissue electrolyte, and oxygen consumption. These values represent mean of % control. n=4 at each vanadate concentration.
● ; aerobic PAH uptake.
□ ; anaerobic PAH uptake.

2) Effect of vanadate on active and passive PAH uptake, tissue electrolyte and oxygen consumption

Active and passive PAH uptake, tissue electrolyte, and oxygen consumption by renal cortical slices were measured in the presence of varying concentrations of vanadate ranging from 10^{-6} to 10^{-2}M(Fig. 2). PAH accumulation was inhibited by vanadate in a similar pattern with the inhibition of Na-K-ATPase activity although the range of inhibitory concentration was very high. In vanadate-free medium, PAH S/M ratio was approximately 7.02. The ID_{50} for this inhibition was approximately 10^{-4}M and was consistent with Sheikh et al.[73] PAH accumulation was nearly abolished at 10^{-3}M vanadate. Vanadate had no effect on the passive transport of PAH. A pronounced inhibition of Na-K-transport as evidenced by a rise of tissue Na and decrease of tissue K concentrations was observed. In "fresh tissue" the concentration of potassium was 88.39±3.17 mEq/kg wet wt. tissue and of sodium was 57.05±4.37 mEq/kg. A small reduction of renal oxygen consumption was also noted in the concentration range from 10^{-5} to 10^{-4}M. This is attributed to the decrease of Na-K transport which requires sizeable contribution of oxygen consumption of the slices[20]. Initial oxygen consumption (Q_{O_2}) of fresh slices was 0.993±0.07(μl/mg final wet wt./hr) and this result was consistent with that of Burg and Orloff[12] and Sheikh et al.[73] But oxygen consumption at 10^{-3}M vanadate was significantly higher than control ($p<0.01$). This suggests an "uncoupling" effect on oxidative phosphorylation under conditions of strong inhibition of Na-K-transport. Maxild et al.[56] observed the similar results in the experiment using metabolic inhibitors.

3) Effect of incubation time on the inhibitory action of vanadate

In the preliminary experiment in which

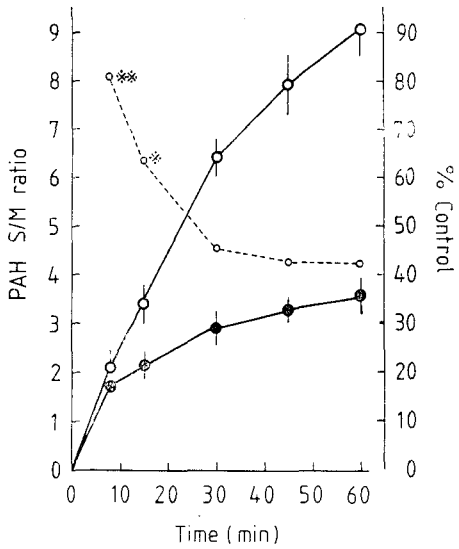

Fig. 3. Effect of vanadate on PAH uptake in rabbit kidney slices as a function of incubation time. These values represent mean±SEM of 4 experiments. Broken line represents % control.
※, significantly different from the extent of inhibition at 30 min(※ ; $p<0.05$. ※※; $p<0.01$)
○ ; control. ● ; 10^{-4}M vanadate.

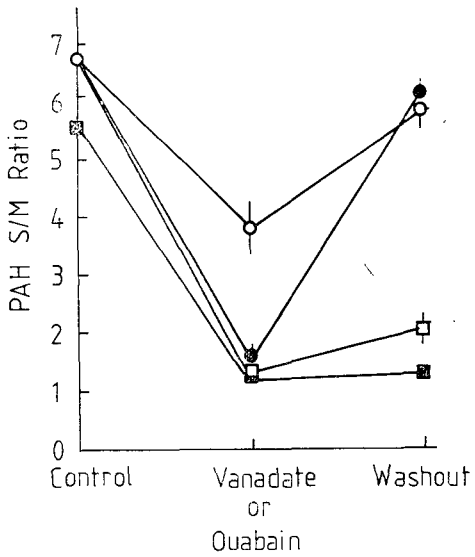

Fig. 4. Effect of vanadate washout on PAH accumulation. These values represent mean± SEM of 3 experiments.
● ; 10^{-4}M ouabain. ○ ; 10^{-4}M vanadate.
□ ; 10^{-3}M vanadate. □ ; 10^{-3}M vanadate, but washout during 75 min.

preincubation was not performed, 10^{-4}M vanadate didn't inhibit the PAH accumulation until 15 min but inhibited the PAH accumulation after 30 min in the same degree. Because it is known that vanadate inhibits the sodium pump from the cytoplasmic side of the enzyme[4,16], accumulates intracellularly via specific anion transport system[16,27] and binds to Na-K-ATPase slowly[45], the above result may be due to the selective permeability of vanadate through basolateral membrane and the slow binding to Na-K-ATPase. Therefore, this experiment was performed after the preincubation for 30 min. The early component of PAH accumulation was inhibited less than the late component in spite of the preincubation in the medium containing 10^{-4}M vanadate. The extent of inhibition of the late PAH uptake was not changed by preincubation(Fig. 3). This result suggests that the rapid uptake of PAH occurs through the mechanism different from the slow uptake process as described previously by Maxild et al.[55] and Sheikh et al.[73] Maxild et al. insisted that the rapid uptake of PAH be dependent on oxidative metabolism, but not affected by Na^+.

4) Reversibility of vanadate inhibition

Figure 4 shows the effect of washout on vanadate and ouabain inhibition of PAH accumulation. The degree of reversibility of the inhibition of PAH uptake was related to the concentration of vanadate in the incubation medium. The inhibition of PAH uptake by 10^{-4}M vanadate and 10^{-4}M ouabain was reversed completely by 45 min washout(Fig. 4).

However, the inhibition of PAH accumulation by 10^{-5}M vanadate was not reversed to an appreciable extent. Smith et al.[75] reported the inhibition of PAH accumulation by 5×10^{-5}M vanadate was not reversed and the amount of vanadate remaining in the renal slices which were rinsed after exposure in 5×10^{-5}M was equal to that present in slices incubated continuously in 1.5×10^{-5}M vanadate. Therefore, they ascribed the irreversibility of the inhibition of PAH uptake by 5×10^{-5}M vanadate to the incomplete washout of vanadate accumulated in slices. But, in the result of Smith et al.[75] PAH accumulation in slices which were rinsed after the exposure in 5×10^{-5}M vanadate was not recovered to PAH S/M ratio in the slices incubated continuously in 1.5×10^{-5}M vanadate, and in this experiment the inhibition of PAH accumulation by 10^{-3}M vanadate was not reversed in spite of longer period of washout (75 min) (Fig. 4). And 10^{-3}M vanadate increased oxygen consumption in slices (Fig. 2). Vanadate also inhibits gluconeogenesis[75] and inhibits glucose-6-phosphatase in rat liver[74]. Irreversibility of 10^{-3}M vanadate, therefore, is not due to incomplete washout but to irreversible cell damage.

5) Effects of the electrolyte composition in the incubation medium

Altering the medium electrolyte concentration of Na^+, K^+ or Ca^{2+} differentially affected the ability of vanadate to inhibit the accumulation of PAH by renal cortical slices. Reduced Na and K were replaced by choline chloride.

(1) K^+ effect

In this experiment, 5×10^{-4}M vanadate was used. Increasing the K^+ concentration from 5 mM to 40 mM at 110 mM Na^+ concentration potentiated the inhibition of PAH accumulation by 5×10^{-4}M vanadate (Fig. 5). Also, as previous studies[23,34] indicated, PAH accumulation in the absence of vanadate increased with increasing medium K^+ concentration.

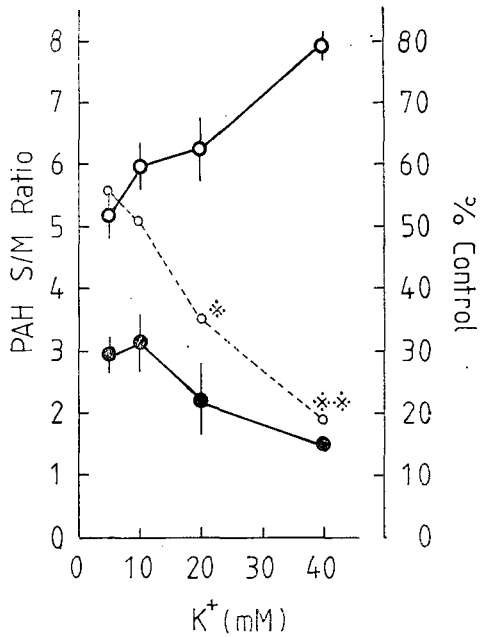

Fig. 5. Effect of altering medium K^+ concentrations on the ability of vanadate to inhibit PAH uptake. Broken line indicates % control. These values represent mean±SEM of 4 experiments.
※ ; significantly different from the extent of inhibition by vanadate at 10 mM K . (※ ; $p<0.05$. ※※ ; $p<0.01$)
○ ; control. ● ; 5×10^{-4}M vanadate.

(2) Na^+ effect

Decreasing Na^+ concentration from 110 mM to 60 mM at 40 mM K^+ concentration potentiated the inhibition of PAH accumulation by 10^{-4}M vanadate (Table 1). Decreasing Na concentration to 30 mM didn't increase further the degree of inhibition of PAH uptake. Decreasing Na^+ concentrations from 110 mM decreased PAH S/M ratio in control.

(3) Ca^{2+} effect

The changes of Ca^{2+} concentration in the incubation medium had no effect on the ability of 10^{-4}M vanadate and 5×10^{-5}M ouabain to

Table 1. Effect of Na^+ on the inhibition of PAH uptake by 10^{-4}M vanadate

Na^+(mM)	Control	10^{-4} vanadate	% Control
110	7.61±0.78	3.82±0.39	50.15±5.74
60	6.07±0.29	2.09±0.20	34.41±3.51*
30	5.24±0.40	2.03±0.15	38.84±2.91*

These values represent mean±SEM of 4 experiments.
*, significantly different from the extent of inhibition of PAH uptake in the presence of 110 mM Na($p<0.05$)

Table 2. Effect of Ca^{2+} on the inhibition of PAH uptake by 10^{-4}M vanadate

Ca^{2+}(mM)	Control	10^{-4}M vanadate	5×10^{-5}M ouabain
1.5	6.99±0.64	3.29±0.23	4.04±0.28
		(47.07)	(57.80)
Ca^{2+}-free	4.77±0.54	2.03±0.29	3.35±0.40
		(42.56)	(61.52)
3.5	6.57±1.03	2.99±0.85	3.96±0.21
		(45.54)	(60.31)

These values represent mean±SEM of 3 experiments.
Values in ( ) indicate % control.

inhibit the PAH accumulation (Table 2). The complete removal of Ca^{2+} from the incubation medium decreased the PAH S/M ratio in control.

6) Effect of pH on the inhibitory action of vanadate

Figure 6 showed the effect of pH on the inhibition of PAH uptake by vanadate in the renal cortical slices. When 10 mM Tris buffer was used, PAH accumulation was increased in accord with pH in the incubation medium in a range from 7.0 to 8.2 (Fig. 6 A). When 10 mM phosphate buffer was used, PAH accumulation showed the maximal value at pH 7.4 (Fig. 6 B). In the presence of vanadate

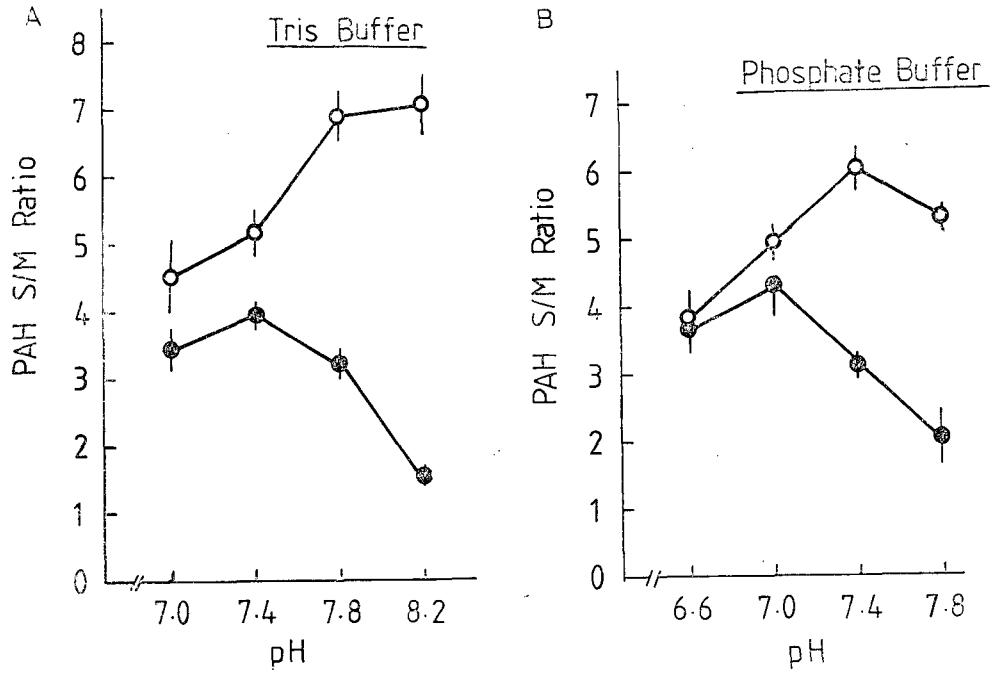

Fig. 6 A and 6 B. Effect of pH on the ability of vanadate to inhibit PAH uptake. These valuses represent mean±SEM of 4 experiments
○ ; control. ● ; 10^{-4}M vanadate.

Table 3. Cumulative effect of vanadate and ouabain on PAH uptake

Inhibitor	Active PAH S/M ratio	Observed inhibition (%)	Calculated cumulative inhibition (%)
Control	5.77±0.28	—	—
10^{-4}M vanadate	2.26±0.26	60.78	—
5×10^{-5}M ouabain	3.24±0.63	43.93	—
10^{-4}M ouabain	1.50±0.31	74.00	—
5×10^{-5}M ouabain+10^{-4}M vanadate	1.32±0.33	77.07	78.01
10^{-4}M ouabain+10^{-4}M vanadate	0.87±0.11	84.92	89.80

Values represent mean±SEM of 4 experiments.

Table 4. Cumulative effect of vanadate and ouabain on Na-K-ATPase activity

Inhibitor	Na-K-ATPase activity (μmol Pi/mg protein/10min)	Observed inhibition (%)	Calculated cumulative inhibition (%)
Control	2.80±0.11	—	—
5×10^{-7}M ouabain	1.64±0.14	41.42	—
10^{-6}M ouabain	1.20±0.09	57.04	—
5×10^{-6}M ouabain	0.50±0.12	82.02	—
10^{-6}M vanadate	1.46±0.18	47.68	—
5×10^{-7}M ouabain+10^{-6}M vanadate	1.02±0.14	63.57	69.35
10^{-6}M ouabain+10^{-6}M vanadate	0.81±0.18	71.23	77.52
5×10^{-6}M ouabain+10^{-6}M vanadate	0.31±0.04	88.99	90.60

Values represent mean±SEM of 4 experiments.

increasing the pH in the incubation medium potentiated the inhibition of PAH uptake by 10^{-4}M vanadate. At the same pH in the incubation medium, the degree of inhibition of PAH accumulation by 10^{-4}M vanadate was different with the buffer used, and 50% inhibition by 10^{-4}M vanadate occurred at pH 7.8 in Tris buffer and at pH 7.4 in phosphate buffer.

7) Additive effect of vanadate and ouabain

To evaluate the interaction of vanadate and ouabain in renal cortex, the effects of the simultaneous administration of vanadate and ouabain on PAH accumulation and Na-K-ATPase activity were observed (Table 3 and 4). The observed values of cumulative inhibition were approximately consistent with the calculated values, and vanadate and ouabain had the additive effect on PAH accumulation and Na-K-ATPase activity.

DISCUSSION

Before discussing the possible mechanisms involved in the inhibitory effects of vanadate observed in this study, some important points regarding the handling of vanadate by cells and the characteristics of vanadate inhibition of Na-K-ATPase need to be emphasized.

First, unlike ouabain, vanadate must first enter cells because it inhibits Na-K-ATPase from the cytoplasmic side of the enzyme[4,16]. Although the mechanism of vanadate transport into renal cells is not known, studies in

erythrocytes have shown that vanadate enters these cells via an anion-exchange mechanism[16]. In addition, the effects of vanadate in other tissues are attenuated by disulfonic stilbene derivatives[7,16]. Second, once inside the cell, a large proportion of vanadate(+V oxidation state of vanadium) is reduced to vanadyl(+IV oxidation state)[13], which is less potent inhibitor of Na-K-ATPase[13,39]. Therefore, two critical determinants of the inhibitory effects of vanadate are its ability to penetrate into cells and the oxidation-reduction equilibrium between the vanadate and vanadyl that forms inside the cell. Third, increasing extracellular potassium concentrations intensifies the inhibition of Na-K-ATPase by vanadate[2~6,9,39], probably increasing the affinity of the enzyme for vanadate[6]. Fourth, decreasing extracellular sodium concentration below physiologic values intensifies the inhibition of Na-K-ATPase by vanadate[2,5,9]. Fifth, vanadate combines reversibly with the Na-K-ATPase[14,62].

In this experiment the inhibition of PAH accumulation by vanadate shared several features in common with above facts. First, PAH accumulation was inhibited by vanadate at approximately 100-fold higher concentration than that for the inhibition of renal Na-K-ATPase activity(Fig. 1 and 2). 2nd, vanadate inhibited PAH uptake reversibly(Fig. 4). 3rd, the inhibition of PAH accumulation by vanadate was enhanced by increasing K^+ and decreasing Na^+ in the incubation medium(Fig. 5 and Tabel 1). Elsewhere, tissue Na^+ and K^+ were affected in the same range with that of the concentration in which PAH accumulation was inhibited(Fig. 2). Vanadate and ouabain inhibited PAH accumulation and Na-K-ATPase activity additively(Table 3 and 4). Also, the direct effect of vanadate on PAH transporter can be excluded because of no effect of vanadate on passive PAH uptake (Fig. 2). All of these facts support the suggestion that vanadate inhibits PAH transport through the inhibition of Na-K-ATPase reversibly at intracellular side and that PAH transport is linked in a direct manner to the function of Na-K-ATPase.

However, several other aspects may be considered. It has been known that vanadate stimulates adenylate cyclase in rat fat cell[71] and cardiac muscle. Podevin and Boumendil-Podevin[64] reported that cyclic AMP and theophyllin inhibits PAH transport in the rabbit kidney cortical slices. However, Westenfelder et al.[89] showed that in rat vanadate stimulates renal adenylate cyclase only in cell membranes and not in the intact animal or tissue slice. Thus, it is unlikely that vanadate inhibits PAH uptake through the stimulation of adenylate cyclase. However, the above possibility can't be excluded completely because cyclic AMP dependent protein kinase may inactivate Na-K-ATPase[10,11].

It has been reported that vanadate inhibits Ca^{2+}-ATPase[68,87,91] and inhibits renin secretion in kidney through intracellular Ca accumulation by the inhibition of Ca^{2+}-ATPase[19,49]. But Ca^{2+} has the reverse effect on PAH uptake and the removal of Ca from incubation medium reduces PAH accumulation[18,31,70]. Matsushima and Gemba[54] insisted that the stimulatory action of Ca on PAH accumulation be in part dissociated from that of Na^+ gradient, although Ca^{2+} exerts partly a stimulatory effect on PAH accumulation by maintaining the Na gradient. In this experiment the change in Ca^{2+} concentration had no effect on the inhibition of PAH uptake by vanadate and ouabain, Na-K-ATPase inhibitors. This suggests that the stimulatory action of Ca^{2+} to PAH accumulation

derives mainly from maintaining the Na^+ gradient.

Wallick et al.[86] and Myers et al.[59] reported that vanadate facilitates the binding of ouabain to the sodium pump in the kidney. Erdmann et al.[30] and Myers and Boerth[58] showed that vanadate stimulates ouabain binding to Na-K-ATPase in the heart. But, Mishra et al.[57] showed that vanadate inhibits ouabain binding to Na-pump in the rat brain. Smith et al.[75] reported that the combined effect of ouabain and vanadate on the inhibition of PAH and TEA accumulation was not significantly different from inhibition by vanadate alone. However, in this experiment vanadate and ouabain showed the cumulative inhibition to PAH accumulation and Na-K-ATPase activity. Because it has been known that vanadate inhibits Na-K-ATPase at site different from the ouabain binding site[46,77], vanadate and ouabain must inhibit Na-K-ATPase additively if vanadate does not inhibit ouabain binding.

Increasing pH in incubation medium potentiated the inhibition of PAH accumulation by vanadate (Fig. 6 A and 6 B). This was consistent with the results observed in the glucose-6-phosphatase of liver by Singh et al.[74] and in the Na-K-ATPase of rabbit kidney by Woo et al. in our laboratory[92]. This can be explained by the suggestion that altered pH may affect the permeability of vanadate through the membrane or its ability to inhibit Na-K-ATPase. The transport process of vanadate through the basolateral membrane of proximal tubule is unknown. Because vanadate is an analogue of phosphate and phosphate inhibits vanadate in tunicate blood cells[27] and vanadate binding to Na-K-ATPase[14], it is possible that vanadate and phosphate are transported by a similar mechanism in the kidney. Because phosphate transport in the luminal membrane occurs through the carrier mediated mechanism which is dependent partly on Na^+ and in the basolateral membrane by the simple diffusion[25], it is more likely that vanadate is transported by simple diffusion process in the slice system, in which tubular lumen is collapsed and transport occurs through the basolateral membrane only. Edwards and Grantham[28] reported that vanadate inhibits PAH uptake more potently in the luminal side than in the basolateral side of the isolated tubule. This supports the above suggestion. Because PKa of $H_2VO_4^-$ is 8.2, the result of this experiment can not be explained with the changes in the permeability of vanadate due to its ionization state if the above suggestion is correct. It is more likely that varying of pH changes the oxidation state, hydrolysis, and the extent of polymerization of vanadium, which induce changes in its ability to inhibit Na-K-ATPase. The same experiment showed that the degree of inhibition of PAH uptake by vanadate at the same pH was different with the buffers used (Fig. 6A and 6B). If vanadate is transported passively, it is unlikely that the interaction of vanadate with buffer itself occurs in the membrane. Tris is known to readily penetrate the cell membrane through a nonionic diffusion mechanism and participate in intracellular buffering[60]. Therefore, there is a possibility that intracellular pH may be changed differentially with the buffer used. Vanadate binds extensibly with intracellular organic compounds[13]. Altered pH may change the protein binding of vandate and its capacity to inhibit Na-K-ATPase. But in this experiment the above possibilities can not be confirmed and further investigations including vanadate flux study in the proximal tubule are required.

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