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DOUBLE DEPENDENCE OF ORGANIC ACID ACTIVE TRANSPORT IN PROXIMAL TUBULES OF SURVIVING FROG KIDNEY ON SODIUM IONS

II. RELATIONSHIP BETWEEN COUNTER-FLOWS OF FLUORESCEIN AND SODIUM ION ACROSS CELL LAYER

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Summary

With the aid of a direct microfluorimetric method a dependence of organic anion (fluorescein) transport into proximal tubules of surviving frog kidney on Na^+-flow in the opposite direction was studied. It was shown that the complete removal of Na^+ from the tubules lumen resulted in inhibition of fluorescein transport of about 30%. After a specific inhibitor of sodium channels, amiloride (10^{-3} M) having been introduced into lumen of the tubules, the fluorescein transport was inhibited to the same extent. Amiloride affects only when Na^+ is present in the tubular lumen. Strophantin K ($5 \cdot 10^{-5}$ M), a specific inhibitor of (Na^+, K^+)-ATPase, reduced fluorescein transport about twice. Substances increasing the 3′,5′-AMP level in cells (theophylline, NaF) and exogenous 3′,5′-AMP inhibited fluorescein transport while substance that decreased the 3′,5′-AMP level intracellularly (carbachol) stimulated it. For realization of these effects Na^+ should be present in proximal tubules lumen.

Thus, the various effects on the Na^+ flow from lumen of the tubules to medium at the level of both the basal and apical membranes alter the rate of organic acid active transport from medium to lumen as a result of changes in the maximum rate of transport (V) with unchanged K_m. It is suggested that the system of Na^+ extrusion from proximal tubules produces peritubular membrane-side (near the membrane) gradient of Na^+ concentration which may be higher than the summary Na^+ gradient between the medium and the cytoplasm. The magnitude of this gradient affects the maximal rate value of Na^+-dependent organic acid transport. So, there is a double dependence of the active transport system on Na^+, and the stages where Na^+ is needed are: (1) the formation of a carrier-substrate-Na^+ complex and (2) the production of substantial membrane-side Na^+ gradient at the expense of Na^+ extrusion from the tubules.

Introduction

In the previous paper it was shown that the fluorescein active transport into proximal tubules of frog kidney is carried out by a typical Na^+-dependent transport system so that an affinity between a carrier in basal membrane and an organic acid depends on Na^+ concentration in the bath medium, as the latter decreases the affinity lowers sharply [1]. As to its mechanism this system is similar to the Na^+-dependent transport systems for amino acids and sugars in various tissues and cells. In addition, the relationship between Na^+ reabsorption from the tubules lumen and fluorescein transport into frog renal proximal tubules was discovered. When Na^+ reabsorption was inhibited by diuretics the fluorescein uptake in the tubules lumen became slower [2].

The Na^+ reabsorption in proximal tubules may be divided into two parts: (1) passive paracellular flow that contributes mainly (about 60%) to total Na^+ flux, and (2) active transcellular flow [3,4]. In this paper we shall refer to the active transcellular Na^+ flow from the tubular lumen to the bath medium. Similar Na^+ flow takes place across other epithelial layers such as amphibian skin and urinary bladder [5,6]. These tissues were studied in this respect more thoroughly than renal proximal tubules. It is shown for the urinary bladder that such agents as ouabain, cyclic AMP (cyclic 3′,5′-adenosinemonophosphate) and substances raising its intracellular concentration (theophylline, isoproterenol, NaF, vasopressin, etc.), as amiloride may influence active Na^+ flow both accelerating it (cyclic AMP) and slowing down the flow (ouabain, amiloride) [7,8]. It is interesting to note that all these three agents affect Na^+ flow in a different way: ouabain inhibits Na^+, K^+-ATPase [9]; cyclic AMP increases sodium permeability of the bladder apical membrane [10] and amiloride blocks sodium permeability channels in the apical membrane [7].

The inhibitory effect of cyclic AMP [11] and strophantin K [12,13] on fluorescein active transport into tubules have been reported already. These results give grounds to suggest that flow of actively reabsorbed Na^+ influences to a certain extent the counter flow of fluorescein. That is why in the present experiments we tried to establish a relationship between the fluorescein active transport from the medium into the tubules lumen and the transcellular Na^+ active transport from the lumen to the medium. We believe that the condition of no small importance for similar experiments is the use of intact renal proximal tubules [1]. It was shown [14] that in kidney cortical slices the cells of proximal tubules were swollen and the tubular lumens were occluded. As a consequence, there was no possibility to study the transcellular fluxes of both Na^+ [14] and *p*-aminohippurate [15] in the slices. Moreover, it is impossible to create a transtubular (transcellular) gradient of Na^+ concentration in slices.

Materials and Methods

The work was carried out on isolated surviving kidneys of the male frogs *Rana temporaria*. The method did not differ from that used in the previous work [1]. For the composition of normal salt solution see ref. 1. Na^+-free medium was obtained by the substitution of NaCl by choline chloride and of $NaHCO_3$ by $KHCO_3$. Media with an intermediate content of Na^+ (3/4, 1/2, 1/4,

1/10 and 1/20 of the Na^+ content in the normal medium) were obtained by mixing appropriate amounts of normal and Na^+-free media. The temperature of solutions was 18—20°C, pH 7.6—7.9. The kidneys were incubated with aeration. The required Na^+ concentration in the lumen of tubules was reached by perfusion of the circulatory system through the aorta arch. When it was necessary that NaF, isoproterenol or amiloride should be in the tubular lumen, these substances were included into a perfusion solution in an appropriate concentration. In experiments on the run out of fluorescein from tubules the kidneys were preincubated with fluorescein ($5 \cdot 10^{-5}$ M) in a normal Ringer's buffer for 15 min and then transferred to a medium without fluorescein, which contained or did not contain Na^+, theophylline, carbachol or strophantin. The extent of the fluorescein run out was estimated by the amount of fluorescein that remained in tubules after 15 and 30 min. The measurements were carried out on a special microfluorimeter with contact lens [16], as was described previously in detail [12]. The fluorescein concentration in tubules was registered as before in working units proportional to photocurrent in the photomultiplayer. The experimental data were subjected to standard statistical treatment; confidence limits were calculated at the 95% significant level. Transport constants were defined by the method described in detail in the previous paper [1].

Results

At the beginning the influence on the fluorescein transport of substances raising the intracellular cyclic AMP concentration (exogenous cyclic AMP, theophylline, isoproterenol, NaF) and of a substance lowering it (carbachol) was investigated. The addition of cyclic AMP (10^{-3} M) or theophylline (10^{-3} M) reduces the uptake of fluorescein ($5 \cdot 10^{-5}$ M) in a tubule after 15 min of incubation on the average by 45 and 34%, respectively. Under the same conditions isoproterenol (10^{-3} M) lowers fluorescein transport by 12%, while carbachol (10^{-3} M) raises it by 10%. The effect of isoproterenol is reinforced if this agent is present only on the side of the lumen (inhibition by 22%). Similarly, NaF (10^{-4} M) added to the bath medium with fluorescein does not affect the fluorescein uptake significantly, but if this agent is introduced only into the lumen it inhibits fluorescein transport on the average by 15%.

These substances influence the fluorescein run out from the tubule into fluorescein-free medium as well (Table I). Theophylline accelerates the run out of fluorescein as compared to the control, whereas carbachol slows it down. Acceleration of the run out was observed also at the action of strophantion. In order to recognize how some of these substances do act on the transport constants, the dependencies of fluorescein transport rate on its concentration in a medium with a constant concentration of theophylline or carbachol were obtained. A similar dependence was studied in the presence of NaF both in the medium and in the tubules lumen, simultaneously. Constants calculated on the basis of these dependencies are presented in Table II. For comparison, similar dependencies were obtained when the medium containing fluorescein only. They are: control I for comparison with experiments with theophylline and carbachol and control II for comparison with NaF. It may be seen that theophylline raises K_m significantly and V nonsignificantly; carbachol raises both

TABLE I

Run out of fluorescein from renal proximal tubules with this acid in the medium with normal Na^+ content and in the Na^+-free (choline chloride) medium in the presence of strophantin, theophylline or carbachol in these media.

Incubation conditions for run out (substances concentration in the medium)	Fluorescein concentration in tubules before run out (Working units)	Run out in the medium with normal Na^+ content				Run out in Na^+-free medium			
		Fluorescein concentration in tubules after incubation				Fluorescein concentration in tubules after incubation			
		15 min		30 min		15 min		30 min	
		Working units	%	Working units	%	Working units	%	Working units	%
Control	404.1 ± 16.0	317.3 ± 12.3	100	246.6 ± 9.3	100	208.5 ± 8.5	100	190.6 ± 8.0	100
Strophantin K $5 \cdot 10^{-5}$ M	404.1 ± 16.0	209.6 ± 11.7	66	151.9 ± 6.8	62	189.0 ± 7.7	91	133.8 ± 5.1	70
Theophylline 10^{-3} M	404.1 ± 16.0	259.1 ± 13.5	82	150.6 ± 7.5	61	165.3 ± 6.6	79	107.8 ± 3.9	56
Carbachol 10^{-3} M	404.1 ± 16.0	348.3 ± 14.7	110	287.3 ± 12.6	116				

TABLE II

INFLUENCE OF THEOPHYLLINE, CARBACHOL AND NaF ON APPARENT MICHAELIS' CONSTANT (K_m) AND MAXIMAL RATE (V) FOR FLUORESCEIN TRANSPORT INTO PROXIMAL TUBULES

Influence	K_m ($\times 10^{-4}$ M)	Confidence limits for K_m ($\times 10^{-4}$ M)	V *	Confidence limits for V *	V/K_m *	Confidence limits for V/K_m *
Control I	1.66	1.13—3.11	45.0	±11.8	27.1	±5.7
Theophylline 10^{-3} M	4.81	3.11—10.59	62.9	±26.4	13.1	±1.7
Carbachol 10^{-3} M	4.03	3.22—5.41	91.7	±16.9	22.8	±1.7
Contol II	2.40	1.97—3.07	63.3	±8.4	26.4	±2.2
NaF $5 \cdot 10^{-4}$ M	1.60	1.18—2.48	39.8	±7.9	24.9	±4.0

* The values are presented in working units.

K_m and V significantly and NaF lowers V significantly and K_m nonsignificantly.

Since amiloride is presumably a blocator of sodium permeability channels in apical membrane it was introduced into the lumen by perfusion. However, it was found that amiloride added to the bath medium only inhibits the fluorescein transport as well. After a 15 min-incubation with fluorescein ($5 \cdot 10^{-5}$ M) and amiloride (10^{-3} M) the average content of fluorescein in tubules decreased from 181.8 ± 7.6 of working units in the control (fluorescein only) to 140.9 ± 6.3 of working units. Therefore it was necessary to check whether amiloride influences the fluorescein transport on the side of basal membrane where it is during perfusion. For this purpose the fresh-excised kidney was placed into an amiloride solution for 6—8 min (duration of perfusion) and then without special washing out it was slightly dried on the paper filter (as was always done before placing the kidney into the incubation medium) and transferred into the incubation medium with fluorescein ($5 \cdot 10^{-5}$ M) for 15 min. As a result an average content of fluorescein in tubules was 177.0 ± 6.7 of working units which is practically equal to that in control (181.8 ± 7.6). Thus it may be assumed that the effects obtained after perfusion with amiloride were the results of its action on the lumen side only.

From Fig. 1 it is seen that amiloride inhibits fluorescein transport and the inhibitory effect of amiloride depends on the concentration of this agent in the tubular lumen. A significant decrease of the transport is observed beginning at the amiloride concentration $2.5 \cdot 10^{-4}$ M. As follows from the time course of fluorescein transport, when amiloride is present in the lumen (Fig. 2) the effect of amiloride on the fluorescein transport is not weakened 15 min after the termination of the perfusion.

Then the action of theophylline, strophantin, NaF and amiloride on the fluorescein transport was investigated at various combinations of Na^+ content in the medium and in the lumen. The results of these experiments are presented in Tables III and IV. From both tables one can see that fluorescein transport depends to a great extent on the content of Na^+ in the medium, but the absence of Na^+ in the lumen only leads to inhibition of fluorescein uptake by

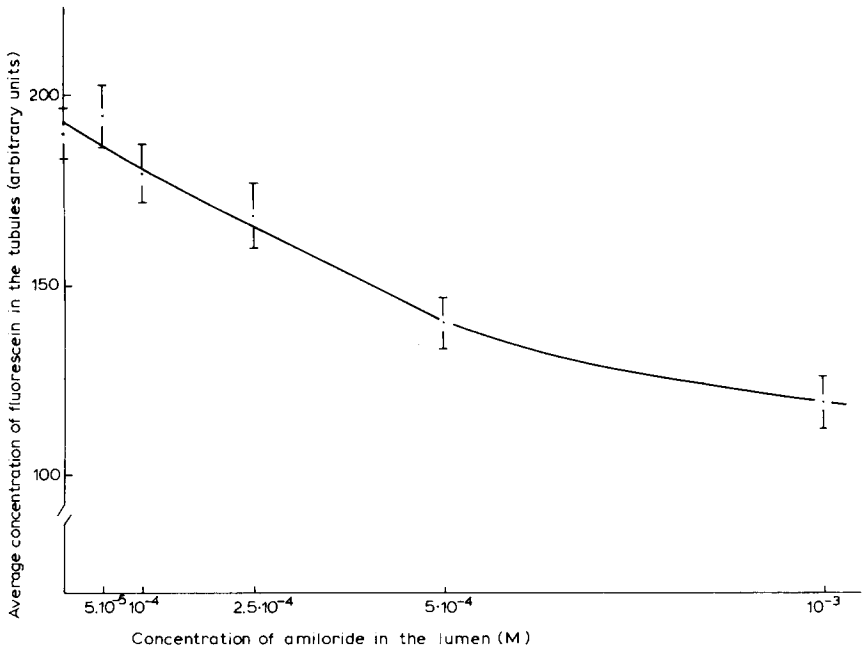

Fig. 1. Action of amiloride introduced into the lumen on the fluorescein uptake in proximal tubules. Fluorescein concentration in the medium is $5 \cdot 10^{-5}$ M. Time of incubation 15 min. The vertical lines show the 95% confidence limits for each value.

25—31%. The action of theophylline is more dependent on the Na^+ content in the lumen than in the medium. Strophantin has a greater inhibitory effect on the fluorescein transport when Na^+ is present in the lumen (Table III, columns 1—4). When Na^+ is absent from the lumen the extent of inhibition by strophan-

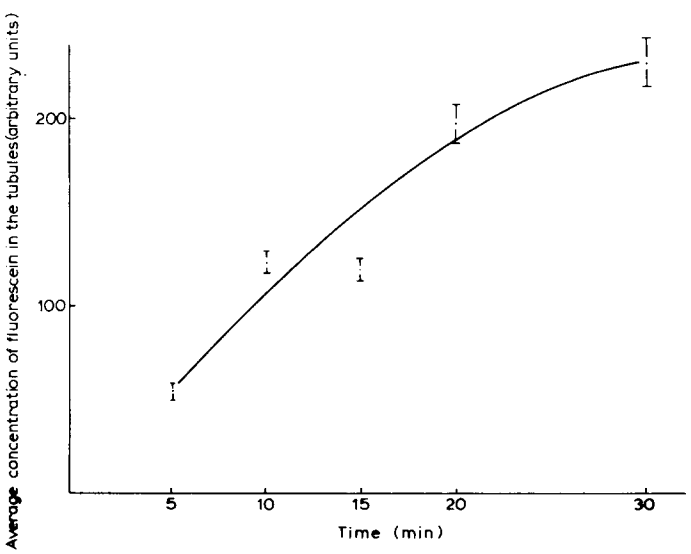

Fig. 2. Time course of fluorescein transport by proximal tubules in the presence of amiloride in the tubular lumen. Amiloride concentration in the lumen is 10^{-3} M. Fluorescein concentration in the medium is $5 \cdot 10^{-5}$ M.

TABLE III

ACTION OF THEOPHYLLINE AND STROPHANTIN ON FLUORESCEIN TRANSPORT IN PROXIMAL TUBULES IN THE ABSENCE OF Na^+ IN THE BATH MEDIUM OR/AND IN THE TUBULES LUMEN

Average fluorescein concentration in tubules is given in working units (mean ± confidence limits) and as % to correspondent control. Na^+ in the medium and in the lumen either is present in normal concentration (113.4 mM/l) or is absent (0). Fluorescein concentration in the medium is $5 \cdot 10^{-5}$ M. Time of incubation 30 min. n.s., the difference with the correspondent control is not significant.

No.	Substances concentration in the medium	Na^+ content			
		Medium 113.4 mM Lumen 113.4 mM	Medium 113.4 mM Lumen 0	Medium 0 Lumen 113.4 mM	Medium 0 Lumen 0
1	Control	370.6 ± 17.3 100%	279.8 ± 10.2 100%	167.3 ± 6.1 100%	156.2 ± 7.2 100%
2	Theophylline 10^{-3} M	259.8 ± 15.2 70%	248.0 ± 7.3 89%	135.0 ± 5.6 81%	155.6 ± 4.6 n.s. 98%
3	Strophantin $5 \cdot 10^{-5}$ M	200.0 ± 8.6 54%	217.8 ± 7.6 78%	112.3 ± 4.7 67%	125.9 ± 3.7 80%
4	Theophylline 10^{-3} M Strophantin $5 \cdot 10^{-5}$ M	172.4 ± 7.6 46%	213.9 ± 7.8 76%	96.9 ± 3.6 57%	119.5 ± 4.0 75%

TABLE IV

ACTION OF AMILORIDE, STROPHANTIN AND NaF ON FLUORESCEIN TRANSPORT INTO PROXIMAL TUBULES IN THE PRESENCE OR/AND IN THE ABSENCE OF Na^+ IN THE BATH MEDIUM AND IN THE LUMEN

Average fluorescein concentration is given in working units (mean ± confidence limits) and as per cent of the correspondent control. Na^+ concentration in the medium or in the lumen is either normal (113.4 mM) or is equal to zero (0). Amiloride and NaF was introduced into lumen by perfusion. Fluorescein concentration in the medium is 10^{-4} M. Time of incubation 15 min. n.s., the difference with the correspondent control is not significant.

No.	Substances concentration in the medium	Na^+ content			
		Medium 113.4 mM Lumen 113.4 mM	Medium 113.4 mM Lumen 0	Medium 0 Lumen 113.4 mM	Medium 0 Lumen 0
1	Control	317.7 ± 10.8 100%	217.6 ± 11.9 100%	163.7 ± 6.4 100%	150.4 ± 7.3 100%
2	Amiloride 10^{-3} M	228.7 ± 10.1 72%	222.2 ± 11.6 102% n.s.	145.7 ± 6.3 89%	154.3 ± 8.2 103% n.s.
3	Amiloride 10^{-3} M Strophantin $5 \cdot 10^{-3}$ M	217.7 ± 8.6 68%	180.0 ± 8.8 83%	128.0 ± 5.6 79%	147.7 ± 7.0 98% n.s.
4	Amiloride 10^{-3} M NaF $5 \cdot 10^{-4}$ M	197.4 ± 7.4 62%	210.0 ± 8.3 96% n.s.	149.8 ± 7.6 91%	143.1 ± 7.1 95% n.s.

TABLE V

INHIBITORY EFFECT OF THE ABSENCE OF Na^+ IN THE LUMEN ON THE RATE OF FLUORESCEIN TRANSPORT INTO TUBULES AT VARIOUS CONCENTRATIONS OF FLUORESCEIN IN THE MEDIUM

Concentration of fluorescein in the medium (M)	10^{-5}	$1.25 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	$5 \cdot 10^{-5}$	$8 \cdot 10^{-5}$	10^{-4}	$1.25 \cdot 10^{-4}$	$2 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$
% of inhibition	24.9	23.6	34.2	9.6	19.2	24.8	6.2	16.8	22.6	31.3

tin does not depend on the Na^+ content in the medium. Strophantin reinforces the theophylline effect at combined incubation only if Na^+ is present in the lumen (Table III, lines 3 and 4). Amiloride acts as an inhibitor of fluorescein transport only in the presence of Na^+ in the lumen (Table IV). Strophantin reinforces the amiloride action when the medium does not contain Na^+. In the presence of Na^+ in the medium and its absence in the lumen, strophantin and amiloride produce a significant inhibitory effect (Table IV, column 2, lines 2 and 3). NaF adds to the inhibitory action of amiloride only in the presence of Na^+ both in the medium and in the lumen simultaneously (Table IV, column 1, lines 2 and 4). When amiloride is introduced into the lumen in the absence of Na^+ both in the medium and in the lumen neither the strophantin nor the NaF effect is observed (Table IV, column 4).

Finally, the influence of Na^+ concentration in the lumen on the rate of fluorescein transport from the medium with the normal Na^+ concentration was studied. Fig. 3 shows that the fluorescein uptake does not depend on the Na^+ concentration until the latter makes up 1/4 of Na^+ concentration in the medium. Then the transport rate decreases and after that does not change until complete removal of Na^+ from the lumen. For such a situation when Na^+ was omitted from the lumen, but where the medium contained the normal amount of Na^+, transport constants were defined. They are $K_m = 1.86 \cdot 10^{-4}$ M (from $1.27 \cdot 10^{-4}$ M to $3.46 \cdot 10^{-4}$ M) and $V = 55.5 \pm 9.6$ (working units/min) in the absence of Na^+ in the lumen as compared to $K_m = 2.07 \cdot 10^{-4}$ M (from $1.55 \cdot 10^{-4}$ M to $3.13 \cdot 10^{-4}$ M) and $V = 76.9 \pm 17.2$ in the control. Thus the absence of Na^+ from the lumen results in the decrease of both K_m and V by 10 and 28%, respectively. It is seen that these changes are not significant at the 95% significant level, although V (but not K_m) decreases significantly at the 80% sig-

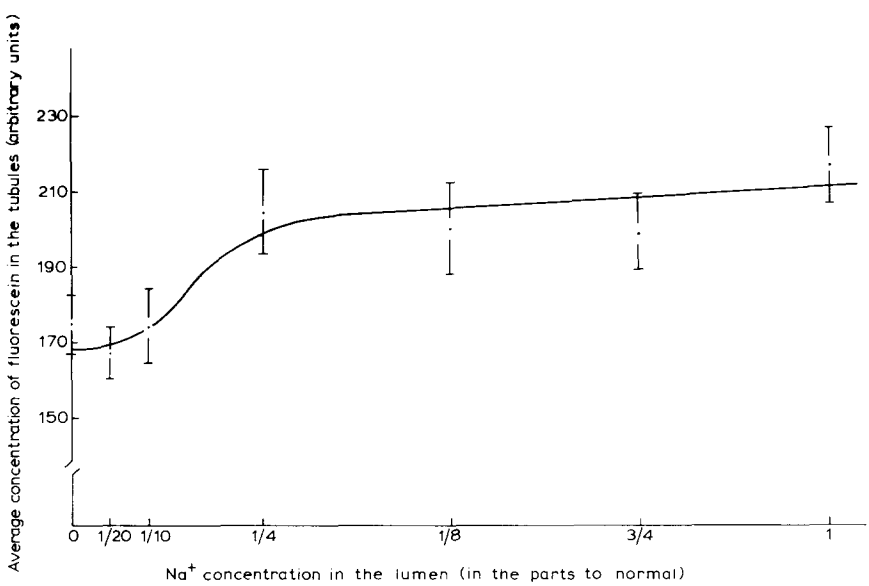

Fig. 3. Influence of Na^+ concentration in the tubular lumen on the fluorescein uptake in proximal tubules. Fluorescein concentration in the medium is $5 \cdot 10^{-5}$ M, time of incubation 15 min.

nificant level. It follows from Table V that at all fluorescein concentrations in the medium the absence of Na^+ from the lumen causes the inhibition of fluorescein transport in the tubules. The average extent of the transport inhibition is 21%.

Discussion

We showed earlier that Na^+ from the medium participates in the formation of a transport complex by changing affinity between a carrier and fluorescein [1]. This evidence is supported by the results of this study. In fact, the fluorescein transport in the absence of Na^+ from the medium decreased by 48—55%. The obtained data suggest as well that for retention of the fluorescein transport rate at the normal level the presence of Na^+ in the tubular lumen is required. One of the principal methods for studying the influence of Na^+ reabsorption on the organic acid active transport was, in this work, the creation of an artificial gradient of Na^+ concentration between the tubular lumen and bath medium. For instance, the tubular lumens were perfused by Na^+-free solution and the kidney was transferred to the medium containing 113.4 mM of Na^+, or the luminal content of Na^+ was 113.4 mM whereas the medium was Na^+-free. When interpreting the data obtained in such experiments it is important to take into account the fact that Na^+ gradient mentioned above was created at the same time with the starting of fluorescein active transport (transfer of kidney into the bath medium). During the process of fluorescein transport the transtubular Na^+ gradient, probably, diminished by degrees. When Na^+ is absent from the bath medium and present in the lumen the diminition may occur as a result of transcellular and paracellular Na^+ reabsorption [3,4]; when Na^+ is absent from the lumen only, it probably penetrates from the bath medium into the lumen in a paracellular way. However, the diminition of Na^+ gradient during the incubation may only reduce its action on the fluorescein active transport. Thus, the obtained effects of the flow of reabsorbed Na^+ on the fluorescein active transport in proximal tubules may be underestimated only. The absence of Na^+ from the lumen only results in a significant inhibition of fluorescein transport (25—31%). The medium Na^+ concentration decrease inhibits the fluorescein transport by lowering an affinity between a carrier and an organic acid [1], whereas the decrease of Na^+ in the lumen leads apparently to the lowering of the maximal transport rate (V). Although V lowering in the absence of Na^+ from the lumen is significant at the 80% significant level only, this fact may be considered to be well established since we observed a significant lowering of fluorescein transport rate at all media fluorescein concentrations used. It should be noted here that the average extent of the fluorescein transport rate inhibition (21%) is in good accord with the V change (28%).

Thus we may assume that the slowing of Na^+ active transport from the lumen to the medium across cells results in lowering the fluorescein active transport from the medium to the lumen. When Na^+ is completely absent from the lumen the strongest inhibitory effect (about 30%) is achieved. The lowering of fluorescein transport rate by decreasing the Na^+ concentration in the lumen occurs by leap. Only when Na^+ concentration in the lumen ranges from 1/10 of the normal concentration to zero the rate of fluorescein transport is

decreased significantly. It is not inconceivable that the Na^+-active flow starts to lower from the beginning of the decrease of Na^+ concentration in the lumen, but down to the concentration 1/4, the flow is sufficient to maintain a normal fluorescein transport rate.

It appears that substances influencing the Na^+-active flow across the amphibian skin and urinary bladder epithelium affect in a similar way the Na^+ transport (and consequently the fluorescein transport) across the cells of frog renal proximal tubules. Strophantin K, a specific inhibitor of (Na^+, K^+)-ATPase, exerts a considerable inhibitory effect on the fluorescein transport. Its action is pronounced more drastically when Na^+ is present in the lumen, i.e. when a Na^+ flow takes place. It is known that in proximal tubules (Na^+, K^+)-ATPase is located in the basal membrane [17]. Thus, the inhibition of Na^+ flow across epithelium at the basal membrane level slows down the fluorescein transport into proximal tubules.

Amiloride in proximal tubules blocks sodium permeability channels, evidently in the same manner as in the urinary bladder, i.e. the Na^+ entry into cells across apical membrane since it is efficient only in the presence of Na^+ in the lumen. Its inhibitory effect on the fluorescein transport increases when a concentration of this agent in the lumen is raised. The maximum effect of amiloride on the fluorescein transport under normal conditions coincides practically with that produced in the case of complete Na^+ absence from the lumen (31.5%). Thus, the inhibition of Na^+ flow across epithelium at the apical membrane level slows down fluorescein transport too.

In our experiments amiloride, added only to the medium, inhibited the fluorescein transport. It is likely that this effect of amiloride is similar to that of harmaline on the Na^+-dependent transport of organic acid (*p*-aminohippurate) in dog kidney slices [18]. Harmaline eliminated Na^+-dependent transport by blocking the Na^+ binding sites on an organic acid carrier in the membrane. It is possible that amiloride, a specific blockator of sodium permeability channels, may compete with the medium Na^+ for binding site on the carrier. In any case the action of amiloride in the bath medium is reversible and does not contribute into effects of amiloride on the apical membrane level.

The substances raising the intracellular cyclic AMP level also inhibit the fluorescein transport into tubules. They are: exogenous cyclic AMP secreted by renal proximal tubules [19], isoproterenol and NaF, which are adenylcyclase stimulators [20], and theophylline, phosphodiesterase inhibitor [20]. Isoproterenol and NaF are more potent as inhibitors if they are introduced into the lumen. This fact agrees with the localization of adenylcyclase in the apical cell membrane [21,22].

On the other hand, carbachol, a phosphodiesterase stimulator [23], which lowers the intracellular cyclic AMP level, stimulates fluorescein transport. Thus, it may be assumed that if a substance increases the intracellular cyclic AMP level it would slow fluorescein transport down, and vice versa.

Recently Podevin and Boumendil-Podevin [24] observed a similar inhibitory action of cyclic AMP, dibutiryl cyclic AMP, theophylline and isoproterenol on the *p*-aminohippurate uptake in rabbit renal cortex slices. Dibutiryl cyclic AMP raised K_m without changing V and, therefore, these authors explained their results as a competition between dibutiryl cyclic AMP (and theophylline) and

p-aminohippurate for a carrier. In our experiments, theophylline also raises K_m without changing V, i.e. it affects the Michaelis-Menten parameters as a competitive inhibitor [25]. But we suggest that all the cyclic AMP level changes may simultaneously act on fluorescein transport in some other way which is different from competition for a transport carrier. Had theophylline only been a competitive inhibitor of fluorescein transport, its action on the fluorescein uptake should not have been dependent on the presence of Na^+ in the tubular lumen as well as its action on the fluorescein run out should not be dependent on the time, as it happened with a typical competitive inhibitor, *p*-aminohippurate under such conditions [1]. Thus, theophylline is likely to influence the fluorescein transport due to the competition for a carrier and increase of the intracellular cyclic AMP concentration.

In our experiments the theophylline action depended on the presence of Na^+ in the tubules lumen. Kinne and coworkers [26] showed that cyclic AMP-binding proteinkynase which is assumed to be a regulator of sodium membrane permeability is located in the apical membrane of proximal tubules. That is why it seems to be very probable that theophylline and other agents (isoproterenol, NaF, etc.) change Na^+ permeability of the apical membrane by means of increasing the intracellular cyclic AMP concentration. The extent of inhibition of a combined action of theophylline and strophantin is the greatest when Na^+ is present in the lumen. Therefore, it may be suggested that when intracellular concentrations of cyclic AMP raises, Na^+ permeability of apical membrane increases. It is obvious that changes in the intracellular cyclic AMP concentration affect the organic acid transport indirectly, due to changes in transcellular Na^+ flow.

Let us summarize all the above mentioned. We investigated different cases when Na^+-flow across cells of proximal tubules was influenced at various stages of the transport process. According to the Koefod-Johnsen and Ussing's model [6] Na^+ active transport across epithelial cells may be divided into two stages: (1) entrance of Na^+ into the cell along its electrochemical gradient across apical membrane and (2) Na^+ extrusion from the cell by (Na^+, K^+)-ATPase against its electrochemical gradient across basal membrane. The sodium pump produces Na^+ and K^+ concentration gradients and, moreover, it may be electrogenous also [27]. We suggest that fluorescein active transport across basal membrane is carried out at the expense of both the Na^+ concentration gradient and electric field in the membrane [1,12]. Consequently Na^+-active flow from the lumen to the medium across the tubular cells may be an efficient regulator of the rate of organic acid active transport into tubules.

The moving force of fluorescein transfer across membrane is not the summary Na^+ gradient but the membrane-side gradient of this ion. The membrane-side Na^+ gradient may differ substantially from the summary Na^+ gradient because of the presence of a flow of this cation and of an unstirred layer in peritubular microenvironment [28]. Therefore if one fails to observe a great change in the intracellular Na^+ concentration after treatment this should not be regarded as evidence of unchanged Na^+ concentration gradient across membrane.

Functional relationships between fluorescein flow and Na^+ flow and modes influencing the latter are presented schematically in Fig. 4. Inhibition of (Na^+,

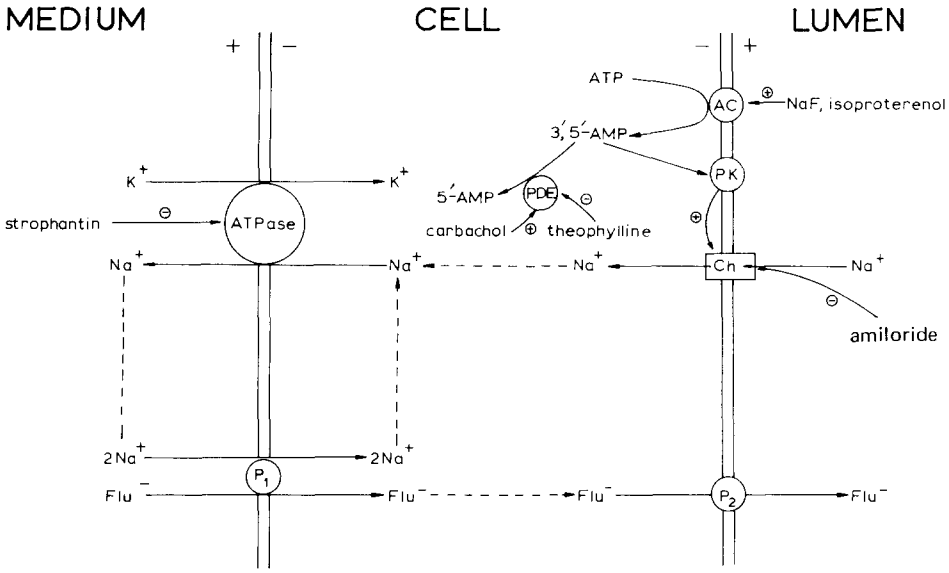

Fig. 4. Transport flows of fluorescein and Na^+ across cells of the frog renal proximal tubules. Abbreviations: ATPase, (Na^+, K^+)-ATPase; P_1, organic acids carrier in basal membrane; P_2 that in apical membrane; Flu^-, fluorescein anion; PDE, phosphodiesterase; AC, adenylecylase ; PK, cyclic AMP binding proteinkynase; Chan, sodium permeability channels in apical membrane; ⊕ means stimulation; ⊖ inhibition or blocking (for channels), "+" and "—" show the direction of electric field in the membrane.

K^+)-ATPase by strophantin in the medium with a normal Na^+ content results in the increase of intracellular Na^+ concentration and in the decrease of membrane-side Na^+ gradient and membrane potential. As a consequence, the fluorescein transport rate is reduced. The increase of an intracellular cyclic AMP level raises apical membrane permeability for Na^+. Hence, the intracellular Na^+ concentration increases, (Na^+, K^+)-ATPase becomes saturated and may not extrude all Na^+ that comes from the lumen. Besides, a high level of intracellular cyclic AMP will probably inhibit (Na^+, K^+)-ATPase (cf. ref. 20). As a result, Na^+ gradient falls and the fluorescein transport decreases. By blocking sodium channels in the apical membrane amiloride lowers intracellular Na^+, and active extruction of Na^+ across basal membrane is thus also diminished. Then the membrane-side Na^+ gradient and membrane potential providing the organic acids transport fall too. The absence of Na^+ in the tubular lumen yields the same results.

Carbachol lowers the intracellular cyclic AMP level and thereby lowers permeability of the apical membrane for Na^+. Therefore it seemed that carbachol should have influenced the fluorescein transport in the same manner as amiloride. In our experiments, however, carbachol stimulate fluorescein transport. As mentioned above cyclic AMP may inhibit (Na^+, K^+)-ATPase [20]. Then the incubation with carbachol may lead to the activation of (Na^+, K^+)-ATPase, and hence, to the increase of both the membrane potential and membrane-side Na^+ gradient.

As shown previously [1] the run out of fluorescein from the tubules also depends on the membrane-side Na^+ gradient. Since strophantin and theophylline accelerate and carbachol slows down this run out, the experiments provide

additional evidence for the membrane-side Na^+ gradient changes by these agents.

Fluorescein transport into the proximal tubular lumen is accomplished due to the work of two membranes (basal and apical), but it is mainly regulated at the basal membrane level. Visual control shows that none of these influences result in any considerable fluorescein excess in the tubular cytoplasm, as one might expect in case the apical membrane is locked for fluorescein [13]. Thus, the basal membrane was a rate-limiting step in fluorescein transport. Therefore, the transport constants (K_m, V) refer to a carrier system in the basal membrane [13]. The fact that the basal membrane is a rate-limiting step in fluorescein transport may be explained as follows: fluorescein transfer across the basal membrane of frog renal proximal tubules demands more energy than that across the apical membrane because fluorescein is transported across the basal membrane against both its concentration gradient and electric field, whereas it moves across the apical membrane also against gradient, but along the field.

It is evident that there are two kinds of dependencies of the organic acids transport in the frog renal proximal tubules on Na^+. Firstly, Na^+ from the medium participates directly in the formation of a transport complex thus increasing an affinity between a carrier and a substrate. Secondly, counter-flow of Na^+ across tubular cells seems to take part in creating a high membrane-side Na^+ gradient across the basal membrane, the magnitude of this gradient regulating the organic acid transport rate. Both membranes (basal and apical) participate in fluorescein uptake in the proximal tubules [1,2], but two types of regulation of the organic acid transport by means of Na^+ are carried out at the basal membrane level.

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