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STIMULATION OF WEAK ORGANIC ACID UPTAKE IN RAT RENAL TUBULES BY CADMIUM AND NYSTATIN

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Abstract—The uphill uptake of a weak organic acid, fluorescein, in superficial proximal tubules of the rat kidney was stimulated by $CdCl_2$ (0.1 mM) or nystatin (20 μM) in the absence of metabolic substrates in the incubation medium. The stimulation could be observed during the initial period of incubation (up to 30 min) only and was prevented completely by ouabain (0.1 mM), fluoroacetate (1 mM), malonate (10 mM), α-cyano-4-hydroxycinnamate (0.1 mM), phenylpyruvate (1 mM), D-malate (2 mM) or phenazine methosulfate (20 μM). In the renal cortex fragment suspension, both Cd^{2+} and nystatin increased the ouabain-sensitive, basal oxygen consumption and inhibited the rate of glucose production from pyruvate, but not from lactate. In the presence of lactate (0.5–5 mM) in the incubation medium, Cd^{2+} and nystatin rather inhibited fluorescein uptake, while externally added pyruvate did not influence their stimulatory effects. Taken together, these data suggest that both activation of the tricarboxylic acid cycle and export of reducing equivalents from the mitochondria to the cytosol are necessary for the stimulatory effects of Cd^{2+} and nystatin on the weak organic acid uptake to develop.

Key words: organic acid uptake; Cd; nystatin; Na,K-ATPase; TCA cycle (rat kidney)

Uptake of weak organic anions, such as *p*-aminohippurate (PAH†), in renal proximal tubules (RPTs), which has been studied *in vitro* for four decades as a model for renal secretion of a great number of anionic xenobiotics, is stimulated by various metabolic substrates (for reviews see Refs 1–3). There are some data showing that PAH uptake can also be stimulated by some substances that do not belong to metabolic intermediates, e.g. by verapamil [4] or Ba^{2+} [5]. In addition, we found that Cd^{2+} at concentrations ranging from 5 μM to 1 mM stimulated the uptake of a weak organic acid, fluorescein, in the RPTs of rat [6] and frog [7] kidneys. It was of interest that such an effect could not be mimicked by Cu^{2+} or Hg^{2+} [6], and Zn^{2+}, Ni^{2+}, Co^{2+} or Mn^{2+} [7].

The stimulatory effect of Cd^{2+} on fluorescein uptake was observed during incubation for up to 30 min only; after 45 min of incubation, Cd^{2+} significantly inhibited uptake. Since omission of Na^+ from the incubation medium caused suppression of the Cd^{2+} effect, we assumed that it was mediated by an indirect activation of Na,K-ATPase [6]. This assumption was evoked by the findings showing stimulation of transepithelial Na^+ transport in frog skin as affected by cadmium ions [8, 9]. As Cd^{2+} affects fluorescein uptake in rat RPTs at 20°, when the basal uptake (i.e. in the absence of externally added metabolic substrates) is not dependent on the presence of external Na^+, mechanisms of the stimulatory effect of Cd^{2+} cannot be satisfactorily explained in the terms of any available theoretical models for the transport system under consideration (review, Ref. 10).

Recently, we have investigated the effects of some inhibitors of gluconeogenesis on fluorescein uptake in rat RPTs and concluded that the renal weak organic acid uptake was regulated by the cytoplasmic pyridine nucleotide redox potential [11]. Since Cd^{2+} was found to elevate the lactate/pyruvate ratio in isolated rat hepatocytes [12], we have examined here the influence of inhibitors of gluconeogenesis on its stimulatory effect on fluorescein uptake. For the purpose of comparison, we used a polyene antibiotic, nystatin, which has a known stimulatory effect on oxygen consumption by RPT cells following indirect activation of Na,K-ATPase (review, Ref. 13). The results support the hypothesis that the stimulatory effect of Cd^{2+} (and nystatin) on fluorescein uptake is mediated by Na,K-ATPase activation and involves acceleration of the tricarboxylic acid (TCA) cycle.

MATERIALS AND METHODS

Procedures dealing with preparation of the outermost rat renal cortex slices and rat renal cortex fragment suspension, as well as methods applied to determine fluorescein uptake by the superficial RPTs in the slices and the rates of glucose production and oxygen consumption in the suspension, have been described recently [11].

Briefly, the work was carried out on the kidneys of male Wistar rats weighing 180–250 g. The outermost cortex slices (0.5–0.8 mm, 50–80 mg) were prepared with the aid of a razor blade and preincubated at 20–22° for 60 min in aerated, substrate-free physiological buffer containing (mmol/L): NaCl 104.7; KCl 15.3; $CaCl_2$ 1.5; $MgSO_4$ 2.5;

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† Abbreviations: PAH, *p*-aminohippurate; RPT, renal proximal tubules; TCA cycle, tricarboxylic acid cycle; PMS, phenazine methosulfate; T/M, tubule/medium.

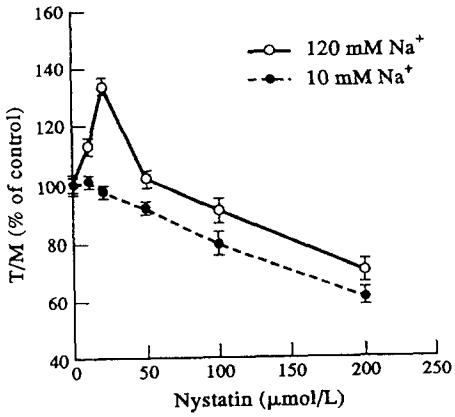

Fig. 1. Dependence of fluorescein uptake on nystatin concentration in the incubation medium. Slices were preincubated in aerated, substrate-free standard buffer at 20° for 60 min and then incubated at 20° for 20 min in the medium containing 120 or 10 mM of Na^+. Each point represents the mean of 120–160 individual records on slices from three to four rats. The vertical lines show the 95% confidence limits. T/M values of the basal fluorescein uptake were 2.68 ± 0.07 (120 mM Na^+ medium) and 2.64 ± 0.06 (10 mM Na^+ medium).

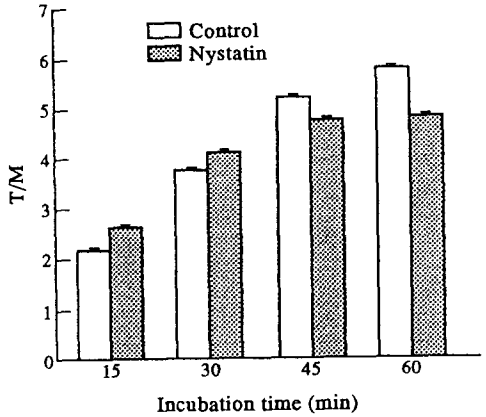

Fig. 2. Time course of fluorescein uptake in the presence of nystatin (20 μM). Slices, preincubated as described in the legend to Fig. 1, were incubated in the standard medium with 0.05 mM fluorescein for 15, 30, 45 or 60 min. Each bar represents the mean of 120–160 individual records on slices from three to four animals. The vertical lines show the 95% confidence limits.

$NaHCO_3$ 3.6; NaH_2PO_4 3.3; Na_2HPO_4 4.8; pH 7.1–7.3 (20°).

For fluorescein uptake measurement, the slices were incubated at 20–22° (usually for 20 min) in 10 mL of fresh, aerated medium of the same content supplemented with 0.05 mM fluorescein. In a few experiments, an incubation medium with a low Na^+ concentration was used, which contained (mmol/L): NaCl 10; choline chloride 110; $CaCl_2$ 1.5; $MgSO_4$ 2.5; $KHCO_3$ 3.6; KH_2PO_4 3.3; K_2HPO_4 4.8; pH 7.0–7.2 (20°).

After incubation, the slices were washed out in the standard solution, and the amount of fluorescein accumulated in the superficial convoluted RPTs was measured using a laboratory-built microfluorimeter with a contact objective lens. On the surface of each slice, the luminescence intensity in 40 different RPTs was measured; the intensity of background luminescence was subtracted. The measurements were repeated on slices from three to four animals, so that each point represents the mean of 120–160 individual records. The uptake data presented as means ± 2 SE were expressed in the form of normalized concentration ratios, T/M (tubule/medium), the T/M value of equilibrium fluorescein accumulation during incubation at 2–4° for 90 min being considered as unity.

The tubule fragment isolation technique without treating the tissue with proteases was adopted from Guder *et al.* [14]. The cortex fragment suspension (final content 4–8 mg protein/mL) was incubated at 20–22° for 30 min in flasks containing 1 mL of the standard physiological buffer (see above for composition) containing 0.5% bovine serum albumin (fraction V, de-fatted, Sigma Chemical Co., St Louis, MO, U.S.A.) in a shaker at 70 cycles/min.

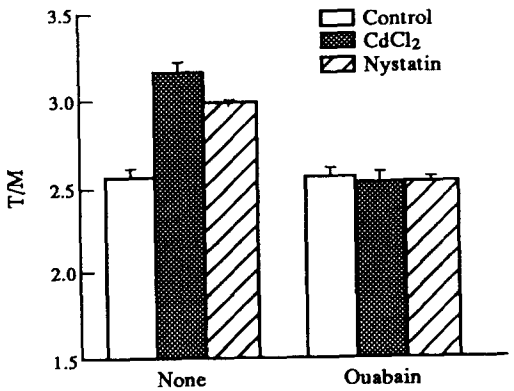

Fig. 3. Influence of ouabain (0.1 mM) on the effects of Cd^{2+} and nystatin on fluorescein uptake. Slices preincubated as described in the legend to Fig. 1 were incubated for 20 min in the aerated, substrate-free standard medium with 0.05 mM fluorescein. Concentrations: $CdCl_2$, 0.1 mM; nystatin, 20 μM.

The incubation was stopped by adding 0.1 mL of ice-cold 30% $HClO_4$. After neutralization and centrifugation, glucose content in clear extracts was determined by the glucose oxidase technique. The pellets were used for measurements of protein content according to a modification of Lowry's method [15].

Oxygen consumption was measured polarographically at 20–22° with a Clarke-type electrode. Aliquots of the cortex fragment suspension (final content 1–2 mg protein/mL) were introduced into the chamber equilibrated with air. Inhibitors were added to the chamber as concentrated aqueous stock

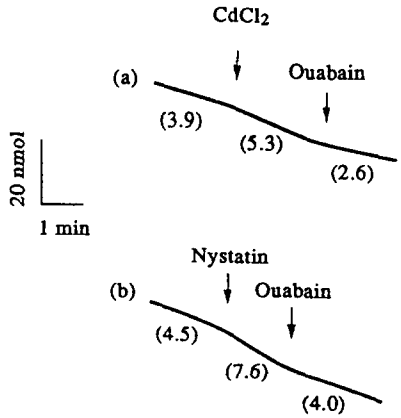

Fig. 4. Oxygen consumption by rat renal cortex fragment suspension as affected by Cd^{2+} (a) and nystatin (b) in the absence of externally added metabolic substrates. Additions (final concentrations): $CdCl_2$, 0.5 mM; nystatin, 0.1 mM; ouabain, 0.1 mM. Tissue concentration was about 1.5 mg protein/mL. The values for basal and altered oxygen consumption (in nmol/min/mL of incubation medium) are in parentheses.

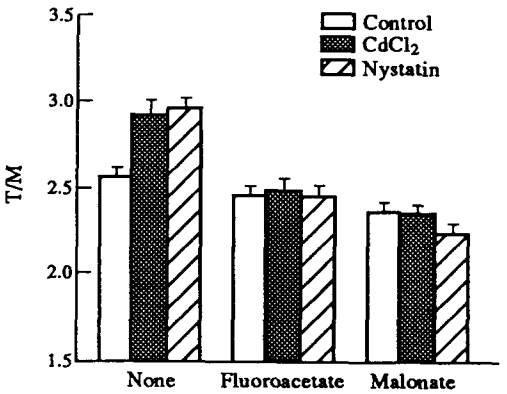

Fig. 5. Influence of fluoroacetate (1 mM) and malonate (10 mM) on the effects of Cd^{2+} and nystatin on fluorescein uptake. Conditions as in the legend to Fig. 3.

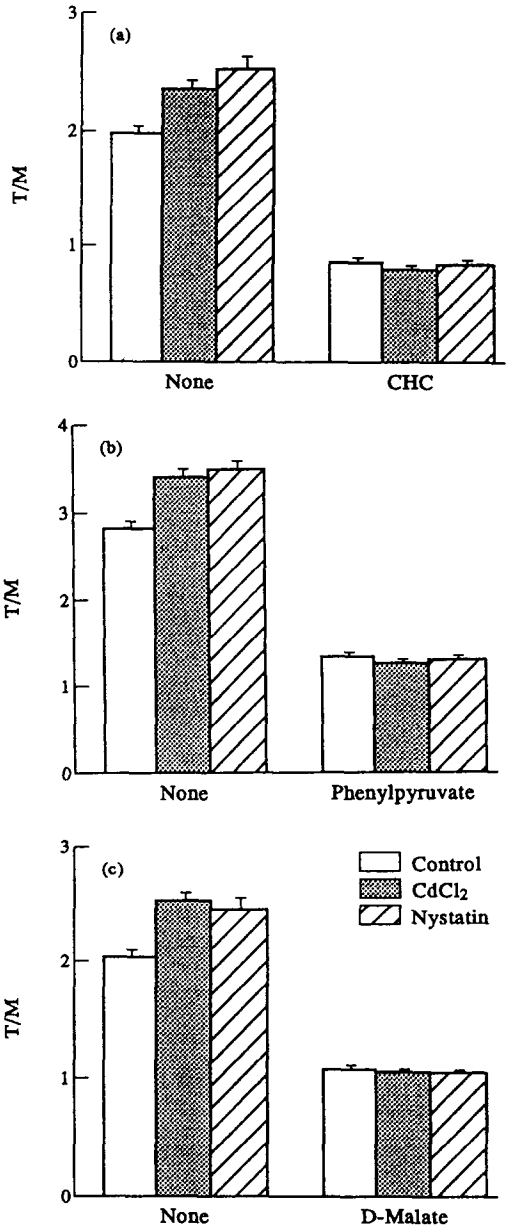

Fig. 6. Influence of α-cyano-4-hydroxycinnamate (CHC; 0.1 mM) (a), phenylpyruvate (1 mM) (b) and D-malate (2 mM) (c) on the effects of Cd^{2+} and nystatin on fluorescein uptake. Conditions as in the legend to Fig. 3.

solutions. The oxygen tension in the chamber was monitored as a function of time.

Chemicals. Fluorescein (disodium salt, uranine, C.I. 45350) was obtained from Koch-Light Laboratories Ltd (Colnbrook, U.K.); quinolinate, α-cyano-4-hydroxycinnamate and D(+)-malate from Sigma; ouabain, monofluoroacetate, malonate, lactate, pyruvate, 2-phenylpyruvate and nystatin (mycostatin; 5215 U/mg) from Serva (Heidelberg, F.R.G.). Other reagents were of commercial grade.

Only aqueous solutions were used throughout the present work. To dissolve nystatin, the pH of a solution was raised to 11.5 (approximately) with 1 N NaOH (then adjusted to pH 7.2 by adding 1 N HCl). Solutions of $CdCl_2$ and nystatin were prepared immediately before use in an experiment.

RESULTS

First, we tested whether nystatin affected the weak organic acid uptake in rat RPTs at all. As seen in Fig. 1, it turned out that nystatin in a narrow concentration range (10–20 μM) significantly stimulated the fluorescein uptake. At concentrations higher than 50 μM, nystatin inhibited the uptake. The stimulatory effect of nystatin was not observed when incubating the slices in the low Na^+ medium. Incubation with nystatin for more than 30 min

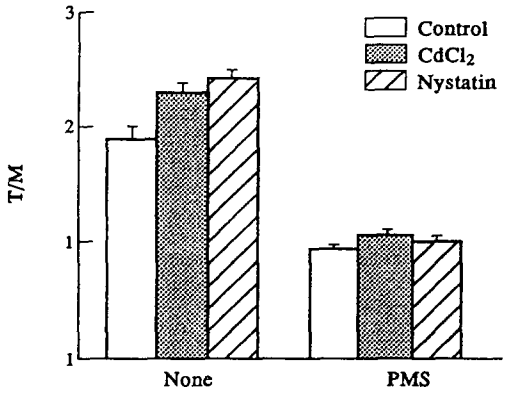

Fig. 7. Influence of PMS (20 μM) on the effects of Cd^{2+} and nystatin on fluorescein uptake. Conditions as in the legend to Fig. 3.

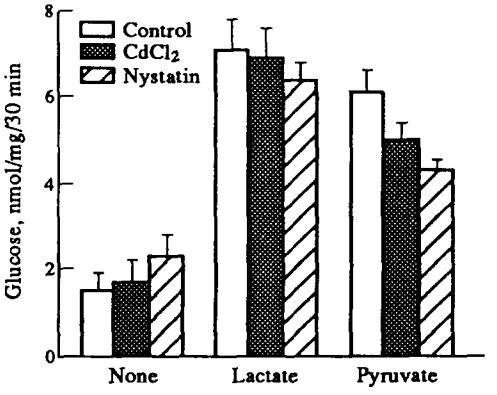

Fig. 9. Effects of Cd^{2+} (0.1 mM) and nystatin (20 μM) on glucose production from lactate (5 mM) or pyruvate (5 mM). Renal cortex fragment suspension was incubated at 20° for 30 min. Data are presented as means ± SE (N = 8).

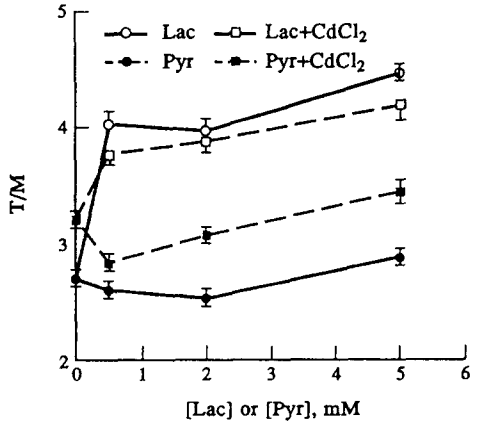

Fig. 8. Effects of Cd^{2+} on fluorescein uptake in the presence of lactate (Lac, 5 mM) or pyruvate (Pyr, 5 mM) in the incubation medium. Incubation in the standard medium; concentration of $CdCl_2$ was 0.1 mM. Other conditions as in the legend to Fig. 3.

resulted in inhibition of the fluorescein uptake (Fig. 2). Preincubation of the slices for 60 min with $CdCl_2$ (0.1 mM) or nystatin (20 μM) caused inhibition of the basal fluorescein uptake after subsequent incubation for 20 min in the substrate- and inhibitor-free, standard medium by 19% and 16%, respectively.

Although ouabain by itself did not affect fluorescein uptake in the rat RPTs at 20°, it prevented the stimulatory effects of Cd^{2+} and nystatin (Fig. 3). Ouabain also blocked the stimulatory effects of Cd^{2+} and nystatin on oxygen consumption by the renal cortex fragment suspension incubated in the substrate-free medium (Fig. 4).

Since the stimulation of ouabain-sensitive respiration by Cd^{2+} and nystatin was probably caused by activation of the TCA cycle, the influence of fluoroacetate and malonate on the stimulatory effects of Cd^{2+} and nystatin on fluorescein uptake was further investigated. It is seen in Fig. 5 that Cd^{2+} and nystatin failed to stimulate the fluorescein uptake in the presence of fluoroacetate or malonate. (Note that in the control neither fluoroacetate nor malonate inhibited significantly the fluorescein uptake.)

It turned out that stimulation of the fluorescein uptake by Cd^{2+} or nystatin was suppressed by the gluconeogenesis inhibitors which diminished both the fluorescein uptake and the rates of glucose production from lactate and/or pyruvate [11]. So, Cd^{2+} and nystatin failed to stimulate fluorescein uptake in the rat RPTs in the presence of α-cyano-4-hydroxycinnamate (Fig. 6a), phenylpyruvate (Fig. 6b) or D-malate (Fig. 6c). In contrast, quinolinate, not affecting by itself the fluorescein uptake in the absence of externally added substrates [11], did not modify the effects of Cd^{2+} and nystatin on the uptake (data not shown).

The stimulatory effects of Cd^{2+} and nystatin were abolished by an electron donor, phenazine methosulfate (PMS) added to the incubation medium (Fig. 7). After preincubation of the slices for 60 min with PMS (20 μM), neither $CdCl_2$ (0.1 mM) nor nystatin affected the fluorescein uptake: T/M values after incubation for 20 min were 0.89 ± 0.3 (Cd^{2+}), 0.92 ± 0.03 (nystatin), 0.93 ± 0.03 (control) and 1.74 ± 0.07 (control after preincubation without PMS).

When pyruvate was added to the incubation medium, the stimulatory effect of Cd^{2+} on fluorescein uptake did not change, whereas in the presence of lactate, Cd^{2+} not only failed to stimulate the uptake but even inhibited it slightly (Fig. 8). It is noteworthy that Cd^{2+} and nystatin inhibited the rate of glucose production from pyruvate but not from lactate (Fig. 9). Neither Cd^{2+} nor nystatin affected the rate of glucose production from endogenous substrates.

DISCUSSION

The data presented here show that Cd^{2+} and nystatin stimulate *in vitro* fluorescein uptake in rat RPTs in much the same manner. The fact that their

stimulatory effects on the uptake can be abolished by ouabain or by the omission of Na^+ from the incubation medium suggests that Na,K-ATPase is involved.

Stimulation by nystatin of oxygen consumption by RPT cells is considered to be exclusively as a result of the dynamic activation of Na,K-ATPase due to enhanced plasma membrane permeability to Na^+ and/or K^+ [16–18]. However, when studied at subcellular level, Cd^{2+} is known to be a potent inhibitor of oxidative phosphorylation (review, Ref. 19). In addition, Cd^{2+} strongly inhibits the Na,K-ATPase activity in microsomal fractions obtained from both frog skin epithelium [20] and rat kidney [21] (Ostretsova and Nikiforov, unpublished results). At the same time, Cd^{2+} stimulates active Na^+ transport across isolated frog skin [8, 9] and ouabain-sensitive oxygen consumption in the rat renal cortex fragment suspension (present work), which "provides an on-line dynamic index of transcellular Na transport" [17]. Such a discrepancy revealed when comparing effects of Cd^{2+} on subcellular constituents and intact cells during short-term incubations can probably be explained by the assumption that there is a barrier against Cd^{2+} penetration through plasma membranes [20]. Uptake of Cd^{2+} in cells of renal origin is a very slow process that is accompanied by the metal binding at the external surface of the cells [22]. So, it can be concluded that in the present work we deal with the action of extracellular Cd^{2+}, which is supported by the fact that the degree of its effect on fluorescein uptake is not dependent on Cd^{2+} concentrations over the range 5 μM–1 mM [6, 7]. Thus, as Cd^{2+} is found to enhance the permeability of plasma membranes of renal culture cells to Na^+ [23] and K^+ [24], it, just like nystatin, could well be an indirect activator of Na,K-ATPase in intact cells.

The only difference in the effects of Cd^{2+} and nystatin on fluorescein uptake emerges from their dose–effect curves. Cd^{2+} stimulated the uptake regardless of its concentration in the incubation medium up to 1 mM [6], whereas the stimulatory effect of nystatin was observed at concentrations of 10–20 μM only; with concentrations higher than 50 μM being inhibitory. It is likely that Cd^{2+} enhances membrane permeability rather moderately, which may be a consequence of its interaction with some specific sites on the membrane surface ("cadmium receptors" [25]). Nystatin interacts with cholesterol in plasma membranes (cited in Ref. 16), and hence its effect on membrane permeability increases with rising concentration. Evidently, Na,K-ATPase has too limited reserve activity to compensate completely for an excessive nystatin-induced imbalance in the intracellular electrolyte composition.

Activation of Na,K-ATPase in intact RPT cells serves as a signal for activation of the cellular oxidative metabolism, in particular, the rate of the TCA cycle (review, Ref. 26). In the previous paper [11] we concluded that the initial steps of the TCA cycle were not the rate-controlling stage in energization (or regulation) of basal fluorescein uptake. As evidenced by the presented results, the dynamic increase in Na,K-ATPase activity as affected by Cd^{2+} or nystatin gives rise to a situation in which inhibition of the TCA cycle reactions does modify the organic acid uptake.

Cellular respiration stimulation by nystatin is shown to be limited in reducing equivalent delivery to the respiratory chain [16]. In such a case, externally added lactate is preferentially utilized as the oxidation fuel [27]. However, in the present work, Cd^{2+} and nystatin failed to stimulate fluorescein uptake in the presence of lactate over a wide range of its concentrations. In this connection it should be remembered that fluoroacetate (1 mM), which inhibited basal oxygen consumption (i.e. in the absence of exogenous substrates) by the rat renal cortex fragment suspension and prevented its stimulation by lactate, pyruvate or acetate, did not influence either the basal fluorescein uptake nor the stimulatory effects of these substrates on the uptake [11]. It is a general observation that PAH uptake in RPTs does not correlate with the rate of oxygen consumption and consequently with the rate of ATP production, in RPT cells (review, Ref. 2). Thus, it may be concluded that activation of the TCA cycle by Cd^{2+} and nystatin gives rise to stimulation of the fluorescein uptake in a more complicated way than merely accelerating ATP production.

Recently, we have postulated [11] that the weak organic acid uptake in rat RPTs, regardless of the presence of metabolic substrates, is regulated by the cytoplasmic pyridine nucleotide redox potential that is maintained at the expense of an export of reducing equivalents from the mitochondria owing to operation of a "futile" cycle suggested by Janssens *et al.* [28]. The data obtained in the present work show that the development of the stimulatory effects of Cd^{2+} and nystatin on basal fluorescein uptake is possible if: (1) the TCA cycle can be activated (prevented by fluoroacetate and malonate), and (2) the above-mentioned "futile" cycle is in operation (suppressed by gluconeogenesis inhibitors and PMS). These requirements will be fulfilled simultaneously if the TCA cycle discontinued at the malate dehydrogenase (L-malate:NAD^+ oxidoreductase; EC 1.1.1.37) reaction stage is "inserted" into the "futile" cycle under consideration.

The dynamic activation of Na,K-ATPase by nystatin (and by cadmium) resulted in the transition of mitochondria in the RPT cells to a more oxidized state (State III) [16, 29], in which the intramitochondrial reduction of oxaloacetate to malate catalysed by malate dehydrogenase would appear unlikely [with more pronounced inhibition of gluconeogenesis from pyruvate as compared to that from lactate [30] (present work)]. Hence, oxaloacetate formed from pyruvate in the pyruvate carboxylase (pyruvate:CO_2 ligase; ADP-forming; EC 4.1.1.31) reaction will preferentially be used in the citrate synthase (EC 4.1.3.7) reaction, while malate (exporting reducing equivalents from the mitochondria) will be formed in forward reactions of the TCA cycle. In the cytoplasm, malate dehydrogenase oxidizes malate to oxaloacetate that, in turn, is decarboxylated by oxaloacetate decarboxylase (EC 4.1.1.3) to pyruvate, the entry of which into the mitochondria completes the cycle [28].

In our opinion, the mechanisms for the stimulatory

effects of Cd^{2+} and nystatin on the fluorescein uptake in rat RPTs, proposed in the present work, may well provide further insight into the role of intramitochondrial events in regulating renal weak organic acid uptake. In particular, this approach could be helpful in elucidating mechanisms of physiological regulation of the exchange (PAH/α-ketoglutarate) transport (review, Ref. 10).

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