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Interaction of Fluorescein with the Dicarboxylate Carrier in Rat Kidney Cortex Mitochondria[1]

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ABSTRACT

The interaction of the organic anion, fluorescein (FL), with mitochondria in renal proximal tubule cells was investigated. Confocal microscopy was used to demonstrate FL accumulation in mitochondria of intact cells. Phenylsuccinate inhibited the mitochondrial accumulation of the FL analog, carboxyfluorescein (CF) indicating that the dicarboxylate carrier may be involved in the intracellular compartmentation of organic anions. To characterize the interaction, radio-tracer uptake and respiration studies with renal mitochondria were carried out using succinate as a substrate. Respiration measurements in freshly isolated kidney cortex mitochondria revealed that FL inhibited ADP-stimulated and uncoupled respiratory rate, indicating that the organic anion inhibited the availability of succinate as a reducing agent. A similar effect on mitochondrial respiration was found for PAH and phenylsuccinate. FL inhibited ^{14}C-succinate uptake concentration-dependently, and Dixon analysis revealed that the nature of interaction between FL and succinate was competitive. K_i values of 0.5 ± 0.2 and 1.1 ± 0.8 mM were calculated for respiration experiments and tracer uptake studies, respectively. The data demonstrate that FL competitively interacts with a mitochondrial dicarboxylate transporter.

Organic anions that are actively secreted by the kidney, accumulate within cells of the proximal tubule. Various investigations showed that the fluorescent organic anion, FL, is handled by proximal tubules in a way similar to p-aminohippurate, the model substrate for the transport system. The advantage of using FL is that the compound can be visualized within cells and lumen using fluorescence microscopic techniques (Sullivan *et al.*, 1990; Miller *et al.*, 1993; Masereeuw *et al.*, 1994). Confocal microscopic images showed that FL is sequestered in rat proximal tubule cells within vesicular compartments, the major one being mitochondria (Masereeuw *et al.*, 1994).

Our study is concerned with the mechanism by which FL accumulates within mitochondria. So far, nine different anion carriers have been found in the mitochondrial inner membrane and all of these transporters play an important role in the regulation of cellular metabolism. The different carrier systems are closely related and have common substrates (LaNoue and Schoolwerth, 1979; Pedersen, 1993). Other xenobiotics have been shown to interact with mitochondrial anion transport systems. For example, valproic acid noncompetitively inhibits succinate transport (Rumbach *et al.*, 1989). The immunosuppressive agent, cyclosporine A, showed two mechanisms of interaction: a noncompetitive interaction with succinate for binding to succinate-coenzyme Q dehydrogenase, and competitive inhibition with an oxidized form of one of the enzyme components (Lemmi *et al.*, 1990). [Beta]-Lactam antibiotics have been reported to interact with the mitochondrial phosphate carrier. The carrier is acylated by these antibacterial agents, primarily resulting in irreversible injury to this carrier and secondarily to mitochondrial oxidative metabolism (Tune, 1993). In each of these three examples, succinate-stimulated respiration is reduced either directly or indirectly. Measuring succinate stimulated oxygen consumption is a simple way to investigate drug effects on mitochondrial function. The rate of respiration is controlled by the uptake of the dicarboxylate into mitochondria. The principal transporter for uptake into mitochondria is the dicarboxylate carrier, although succinate may also be a substrate for the related tricarboxylate and oxoglutarate carriers (LaNoue and Schoolwerth, 1979). The dicarboxylate carrier catalyzes electroneutral exchange of dicarboxylates and phosphate through the mitochondrial inner membrane. Most of our present knowledge about mitochondrial succinate uptake comes from studies with isolated rat liver mitochondria. The kinetics of succinate uptake appeared to be saturable,

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ABBREVIATIONS: FL, fluorescein; CFDA, carboxyfluorescein-diacetate; CF, carboxyfluorescein; PAH, para-aminohippurate; PTC, proximal tubular cells; EGTA, ethylene glycol-bis-(β-aminoethylether)-N, N,N′,N′-etraacetic acid; BSA, bovine serum albumin.

concentration-, temperature- and pH-dependent, and sensitive to phenylsuccinate. Low concentrations of phenylsuccinate blocks dicarboxylate uptake in isolated mitochondria, but the drug is not transported into the matrix (Quagliariello *et al.*, 1969; Palmieri *et al.*, 1971).

We use confocal microscopy of intact renal proximal tubule cells, and radio-tracer uptake and respiration studies with renal mitochondria to demonstrate that substrates for the renal organic anion transport system competitively interact with the mitochondrial dicarboxylate carrier.

Materials and Methods

Materials. FL, succinic-acid, phenylsuccinic-acid and EGTA were purchased from Aldrich Chemie (Steinheim, Germany) and [2,3-^{14}C]succinic-acid (specific activity 2.2 GBq/mmol) from Du Pont (Boston, MA). CFDA and tetramethylrosamine were from Molecular Probes (Eugene, OR). BSA and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid were obtained from Boehringer-Mannheim (Mannheim, Germany). Sodium pentobarbital was acquired from Apharmo (Arnhem, The Netherlands) and heparin from Organon (Oss, The Netherlands). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Confocal images of isolated proximal tubular cells. Proximal tubular cells were isolated as described in detail previously (Masereeuw *et al.*, 1994). For microscopy, cells were transferred to a Teflon chamber with a glass coverslip floor, containing 1.5 ml of incubation buffer (117.5 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 0.95 mM KH_2PO_4, 22.5 mM $NaHCO_3$, 11.1 mM glucose and 2.5 mM $CaCl_2$). Cells were preincubated for 15 min in presence or absence (controls) of phenylsuccinate (25 μM or 1 mM) or nocodazole (20 μM) at 37°C under an atmosphere of 95% oxygen and 5% carbon dioxide. Subsequently, CFDA was added to the cells reaching a final concentration of 2 μM and incubation took place for 15 min at room temperature. In double labeling experiments, cells were preincubated for 15 min at 37°C under an atmosphere of 95% oxygen and 5% carbon dioxide, then CFDA (2 μM) and tetramethylrosamine (0.5 μM) were added simultaneously. Incubation with both dyes took place for 15 min at room temperature. Confocal fluorescent images were obtained with a Zeiss LSM 410 confocal microscope (Carl Zeiss, Oberkochen, Germany). The system consisted of an inverted microscope, a mixed argon/krypton-ion laser with the 488 and 568 lines as excitation source (intensity 15 mW). The microscope was equipped with a 40× oil immersion objective, exhibiting a numerical aperture of 1.3. For measurement of tetramethylrosamine fluorescence, the 568-nm laser line, a 575-nm dichroic filter and a 590-nm long pass emission filter were used. For measurements of CF, the 488-nm laser line, a 510-nm dichroic filter and 515-nm long pass emission filter were used. Neutral density filters passing 1 or 3% of the light and 20% laser power were used to minimize photobleaching. The photomultiplier gain was adjusted so that the average pixel intensity in the cells was between 40 and 60 on a scale of 0 to 255, and cellular autofluorescence was undetectable. For dual labeling studies, the photomultiplier gain in the CF channel was adjusted so that the tetramethylrosamine signal was not detectable. The software used to obtain the images was Zeiss LSM4 (Carl Zeiss, Oberkochen, Germany). Images were stored on an optical disk recorder and analyzed using a Macintosh computer equipped with image analysis software (Image 1.54, National Institutes of Health). The effect of phenylsuccinate on CF accumulation was measured. To this end, a field containing 20 cells was selected and a single 8-sec scan was collected in confocal fluorescence mode. Of this image the average pixel intensity for each cell was calculated of which the background was subtracted, and the value used was the mean of all measured cells. Of one preparation four fields were measured and averaged.

Isolation of kidney cortex mitochondria. Rat kidney cortex mitochondria were isolated as described by (Cain and Skilleter, 1987), with some modifications. All steps were carried out at 4°C. Briefly, male Wistar-Hannover rats (230–280 g) were anesthetized i.p. with pentobarbital (60 mg/kg). Heparin (125 U/100 g) was administered in the femoral vein. Mesenteric and celiac arteries were ligated and the aorta was cannulated above the renal arteries. The kidneys were perfused with an ice-cold solution containing 140 mM NaCl and 10 mM KCl, the vena cava inferior was ligated and the left renal vein was cut open. After perfusion the kidneys were removed and kept in ice-cold isolation buffer (300 mM mannitol, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 1 mM EGTA, 1 mg/ml BSA, adjusted to pH 7.4). The kidney capsule was removed, medulla was dissected and the cortex was collected in three times tissue weight of isolation buffer. Tissue was homogenized in six strokes by hand in a Potter-Elvehjem homogenizer with Teflon pestle (Braun, Melsungen, Germany). The clearance between pestle and Potter tube was 0.5 mm. The homogenate was filtered through two layers of woven gauzes, and centrifuged for 10 min at 500 × g. Supernatant was collected and centrifuged for 7 min at 11,000 × g. Mitochondrial pellet was washed with isolation buffer and again centrifuged (7 min, 11,000 × g). Final pellet was diluted to a concentration of 5 mg/ml mitochondrial protein in respiration medium (210 mM mannitol, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EGTA, 60 mM Tris-HCl, at pH 7.4) and kept on ice.

Protein content was determined using the BioRad Protein Assay of BioRad (München, Germany) with BSA as the protein standard. The purity of the mitochondrial fraction was assessed by assaying the specific activity of the following marker enzymes, as described previously (Russel *et al.*, 1988): succinate dehydrogenase for mitochondria, Na,K-ATPase for basolateral membranes, alkaline phosphatase for brush-border membranes, acid phosphatase for lysosomes and NADPH-dependent cytochrome-c reductase according to Omura and Takesue (1970) for smooth endoplasmic reticulum. The results of the enzymatic analysis (means ± S.D. of six different preparations, unless indicated otherwise) showed an enrichment in succinate dehydrogenase of 3.4 ± 0.5-fold (n = 18), 1.3 ± 0.4-fold in Na,K-ATPase, 1.4 ± 0.5-fold in alkaline phosphatase, 1.4 ± 0.2-fold in acid phosphatase and 0.9 ± 0.5-fold in NADPH-dependent cytochrome-c reductase. Respiration measurements or tracer uptake experiments were carried out immediately after isolation of the mitochondrial fraction.

Mitochondrial respiration. Oxygen consumption was measured using 1 mg of mitochondrial protein at 30°C with a Clark-type platinum electrode in 2.0 ml of respiration medium, as previously described (Cain and Skilleter, 1987). Succinate (10 mM) was used as the metabolic substrate. The conversion of succinate is directly linked to the process of oxidative phosphorylation, and rotenone (5 μM) was added to block electron transport proximal to succinate entry into the respiratory chain. ADP stimulated respiration (state 3) was measured in the presence of 0.3 mM ADP. Dinitrophenol (44 μM) was used as an uncoupling agent. Dinitrophenol discharges the proton electrochemical gradient across the mitochondrial inner membrane, hereby stimulating oxygen consumption without ATP synthesis. The composition of the solutions used for respiration experiments is given in the figure legends. Respiratory rates are expressed as nanogram atoms of oxygen per minute per milligram of mitochondrial protein (ng atom O/min·mg prot.).

^{14}C-Succinate uptake. Uptake of ^{14}C-succinate into mitochondria was measured in triplicate at 10°C. Incubation was started by the addition of 25 μl of the mitochondrial suspension to 100 μl respiration medium supplemented with antimycine (10 μM), rotenone (5 μM), the desired concentration of succinic acid and a tracer amount of ^{14}C-succinic acid (50 nCi). Uptake was stopped at appropriate time intervals by the addition of 2.5 ml ice-cold stop buffer (190 mM mannitol, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EGTA, 60 mM phenylsuccinic acid, at pH 7.4). Separation of mitochondria and extramitochondrial medium took place through Whatman GF/F fil-

ters, using a rapid vacuum filtration technique (Russel *et al.*, 1988). Incubation tubes and filters were washed three times with 2.5 ml stop buffer. To the filters, 4 ml of scintillation fluid (Aqualuma Plus, Lumac, Schaersberg, The Netherlands) was added. The radioactivity remaining on the filters was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding in the absence of mitochondria. Uptake is expressed as pmol/mg mitochondrial protein.

Data analysis. All data are expressed as means ± S.D. and were performed on at least three different mitochondrial preparations. Statistical differences between means were determined using one-way analysis of variance followed by the least significant difference posttest. Values were considered statistically different when $P < .05$. The kinetic constants of the inhibitory potency of FL on succinate uptake in mitochondria were determined from the initial linear uptakes (10 sec) at various substrate concentrations. Inhibition of FL on succinate uptake was expressed relative to the maximum inhibition achieved with phenylsuccinate, and calculated as:

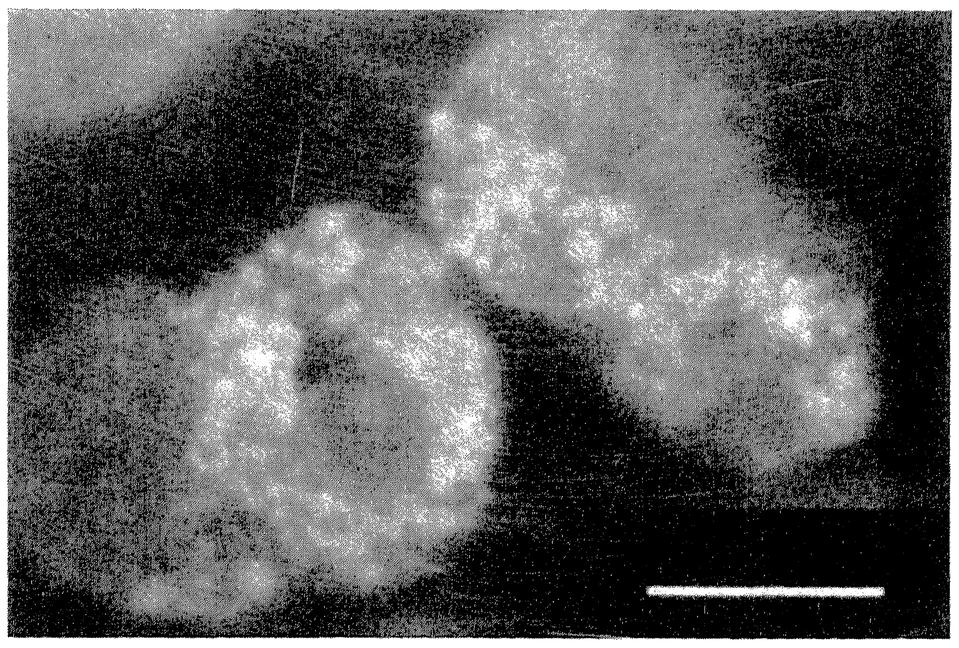
$$V = \frac{V_0 - V_{FL}}{V_0 - V_{PS}} \cdot 100\%$$

where V_0 is the uptake of 100 μM succinate without preincubation with fluorescein, V_{FL} is the uptake of 100 μM succinate determined after preincubation with fluorescein and V_{PS} is the uptake of succinate determined after preincubation with 12.5 mM phenylsuccinate. The log-concentration inhibition curves were analyzed by means of the least squares nonlinear regression program GraphPad Inplot 4.0, assuming a one-site competition model. Transformation of the data according to Dixon showed the nature of inhibition, and allowed calculation of K_i for FL (Dixon, 1953). The K_i value was determined from the average of the intersection points and a standard deviation was derived from the sum of the estimated variance of each intersection point.

Results

Confocal microscopy. CFDA is a nonfluorescent and uncharged analog of FL that enters proximal tubular cells mainly by diffusion. Once inside, the compound is hydrolyzed to CF, an anion, which is highly fluorescent (Dive *et al.*, 1988). Figure 1 shows confocal images of rat kidney PTC incubated with 0.5 μM tetramethylrosamine (upper panel) and 2 μM CF (lower panel). Tetramethylrosamine stains mitochondria (Whitaker *et al.*, 1991) and it is obvious that CF and tetramethylrosamine are localized in the same punctate compartment. This is similar to what was found previously for FL (Masereeuw *et al.*, 1994).

After isolation of rat PTC, there is still a clear distinction between basolateral and brush-border region (fig. 2, upper panel). The darker region in the image is the nucleus, which is located at the basolateral side. The nuclear envelop should be permeable to CF, and the relative fluorescence in this region could be considered as an indication of the level of free drug in cytoplasm. In addition to accumulation of CF in mitochondria, some punctate fluorescence was observed at the brush-border side of the cell compared to the darker nuclear region of the cells. When the cells were preincubated with an inhibitor of mitochondrial dicarboxylate transport, phenylsuccinate, hardly any mitochondrial accumulation could be observed for CF whereas accumulation at the brush-border side increased profoundly (fig. 2, lower panel). Phenylsuccinate inhibited CF accumulation by 65 ± 4% (determined in four different preparations).



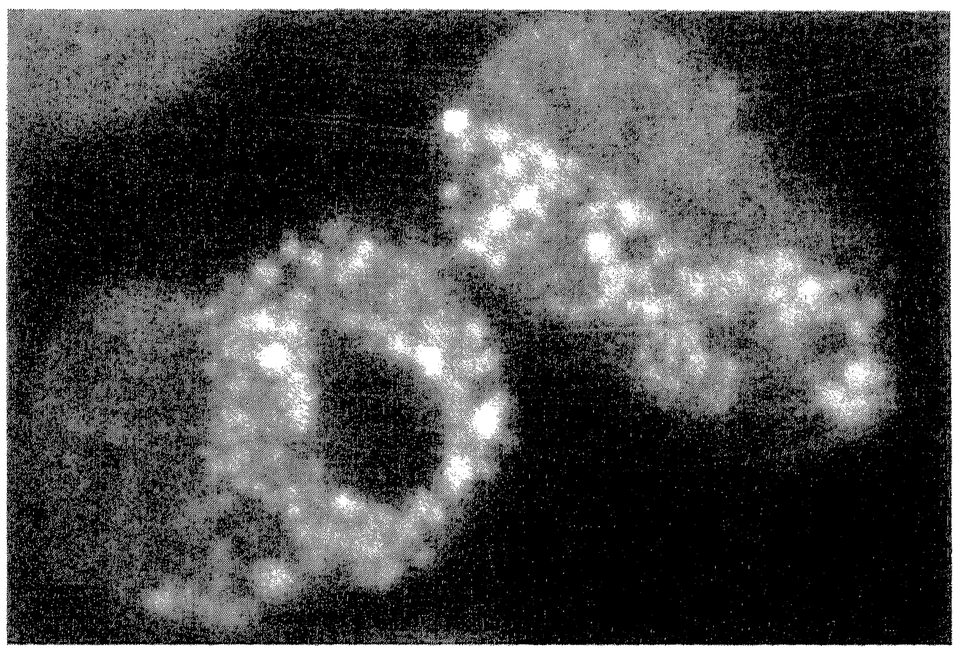
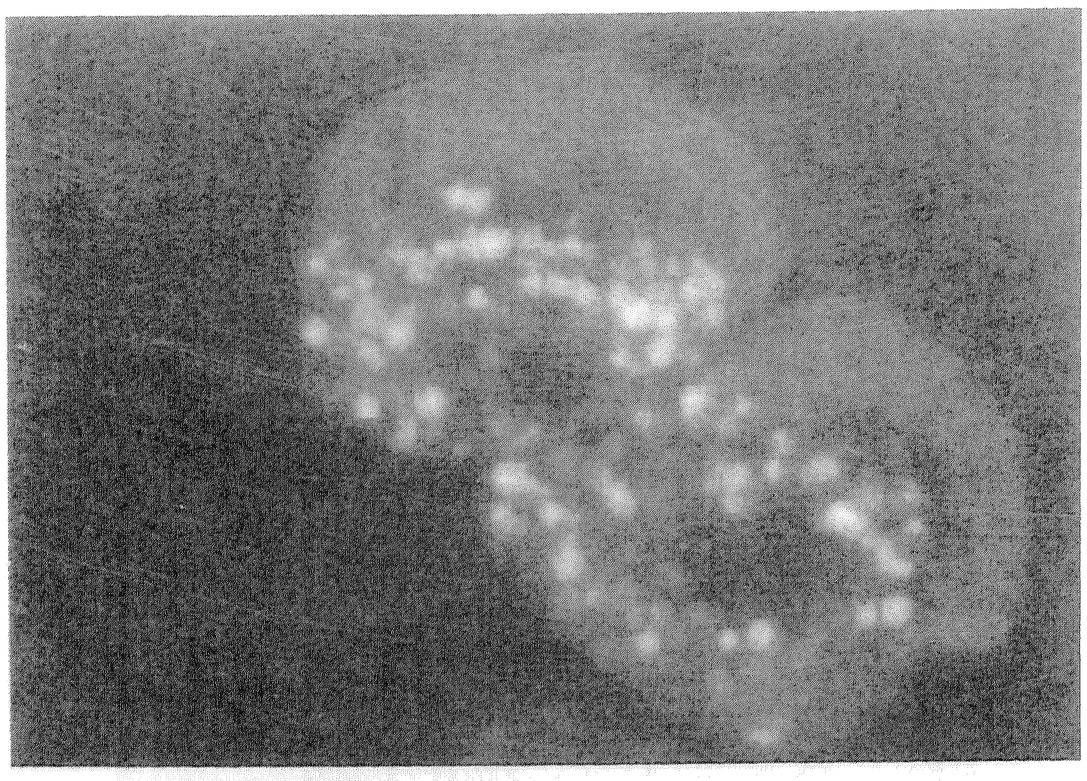


Fig. 1. A representative confocal microscopic image of rat kidney PTC after double-labeling with 0.5 μM tetramethylrosamine (upper panel) and 2 μM CFDA (lower panel). Images were collected as described in "Materials and Methods." Tetramethylrosamine stains mitochondria and the similar accumulation pattern obtained with CF (released from CFDA) indicates that this fluorescent anion is sequestered in the mitochondrial compartment. The polarity of the cells is based on the location of the nucleus and mitochondria, which are basal in tubules. The experiment was replicated in four different isolations. Bar is 10 μm.

Mitochondrial respiration. To determine the nature of the interaction between FL and mitochondria, we measured respiration rates in isolated mitochondria. In figure 3, the control respiratory rate is shown as a function of succinate concentration. A clear concentration-dependent ADP-stimulated respiration (state 3) was observed, whereas the basal respiration (state 2) and recovery respiratory rate after ADP consumption (state 4) were only slightly dependent on the succinate concentration. The concentration of succinate used for most other experiments was 10 mM, which gives maximum state three respiration rates. Cain and Skilleter (1987) demonstrated that respiratory control ratio values (ratio of state 3 over state 4 respiration) of more than 3 for succinate-linked respiration are indicative of intact and well- coupled mitochondria. With 50 mM succinate, the respiratory control ratio value was 4.1 ± 0.2 ($n = 4$), indicating that our mitochondrial preparation was of good quality and tightly coupled.

Table 1 presents the effects of FL and various other organic anions at 100 μM on succinate stimulated respiration. No significant effects of these drugs could be observed on the basal respiration (state 2) of the mitochondrial fraction, although there was a tendency for reduced basal respiration with FL and PAH. After addition of ADP (state 3) FL and PAH significantly reduced respiratory rate, suggesting that the availability of succinate as a reducing agent was decreased. The other organic anions tested did not show any



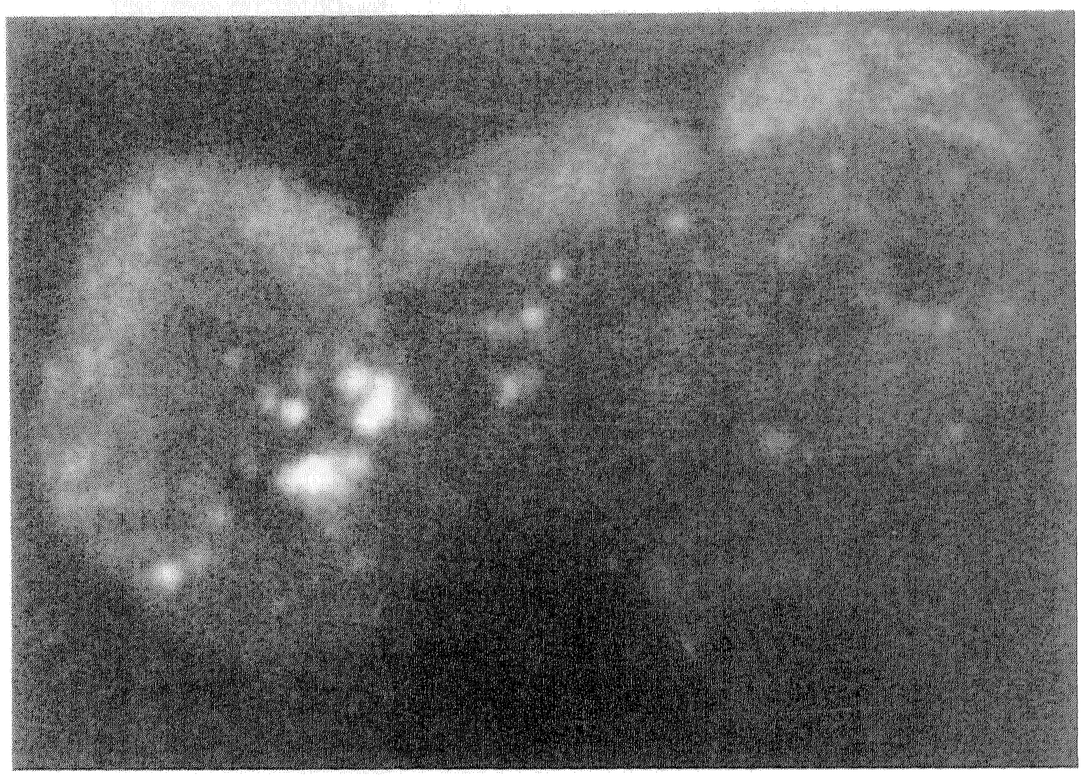


Fig. 2. Two representative confocal microscopic images of rat kidney PTC incubated with 2 μM CFDA, in controls (upper panel) or after preincubation with 1 mM phenylsuccinate (lower panel). Images were collected as described in "Materials and Methods." Controls show the punctate compartment that is additive to mitochondrial CF accumulations. Phenylsuccinate blocks mitochondrial accumulation of CF, resulting in an increase in accumulation in the punctate compartment. The difference in cellular fluorescence reflects changes in gain. The experiment was replicated in four different isolations.

effect on state 3 respiration. Neither state 4 respiration nor the uncoupled rate were affected significantly by any of the agents tested. All drugs tested, except salicylate, significantly reduced respiratory control ratio values. This was a result of either a significant decrease in state 3 (FL, PAH) or a small but not significant increase in state 4 respiratory rate (probenecid, valproate, indomethacin and naproxen).

Respiratory rates measured after preincubation of mitochondria with various concentrations of FL are given in figure 4. Two succinate concentrations were used: 2.5 mM (upper panel) and 10 mM succinate (lower panel). At some concentrations FL inhibited the succinate stimulated respiration significantly; however, a clear concentration-dependent inhibition could not be observed. A plot of the state 3 respiratory rate as a function of FL concentration according to Dixon (1953) is presented in figure 5. The lines intersect above the x-axis and to the left of the y-axis, indicating that FL competitively inhibits succinate-dependent respiration. The K_i value for FL determined from the intersection point was 0.5 ± 0.2 mM.

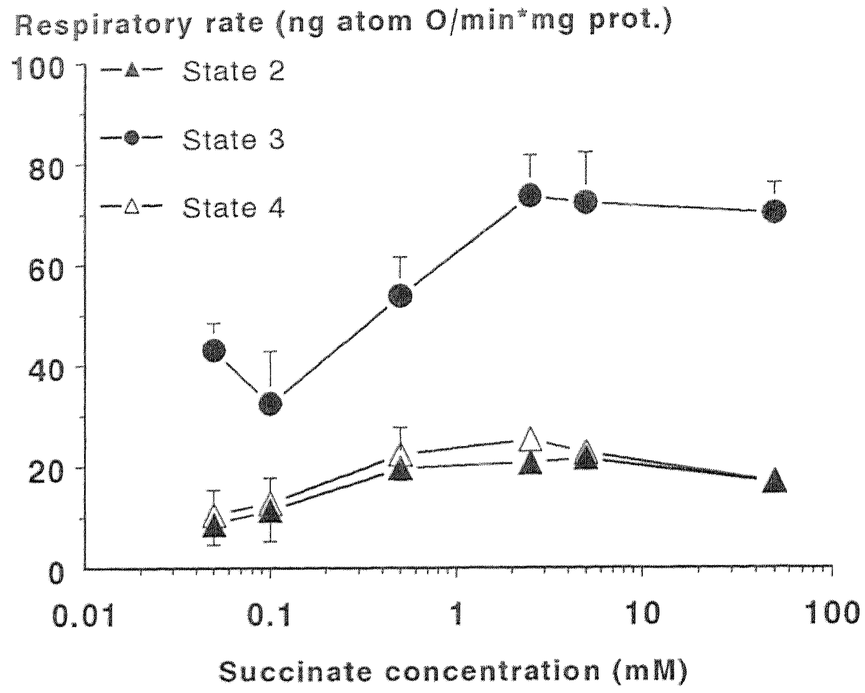


Fig. 3. Respiratory rate of rat kidney cortex mitochondria as a function of different succinate concentrations. Respiration was measured in 2 ml buffer at 30°C, in basal state (state 2), in the ADP-stimulated state (state 3) and after ADP consumption (state 4). Data points are expressed as means ± S.D. of four different isolations.

^{14}C-Succinate uptake. To determine whether FL affected substrate uptake into mitochondria, we measured 500 μM ^{14}C-succinate uptake. Uptake increased linearly with time during the first 2 min, afterwards steady-state was reached (fig. 6). At all times, succinate uptake was significantly reduced by 12.5 mM phenylsuccinate. Initial uptake at 10 sec was inhibited 71 ± 13% (n = 7; P < .001). Succinate uptake values after preincubation with FL were calculated according to equation 1, and a plot of the log-concentration FL *vs* percentage of succinate uptake is presented in figure 7. The succinate concentration was 100 μM, and the concentration at 50% inhibition (IC_{50}) of FL derived from this plot was 0.92 ± 0.03 mM. Concentration-dependence of inhibition by FL was determined for two additional succinate concentrations, and transformation of the data according to Dixon (1953) is shown in figure 8. In accordance with the results of inhibition of respiratory rate, FL inhibited ^{14}C-succinate uptake into mitochondria competitively, and a K_i value of 1.1 ± 0.8 mM could be calculated from the three intersection points.

Discussion

During secretion, drugs that are organic anions accumulate within PTC of the kidney. Although little is known about the intracellular disposition of drugs and their effects on cellular function, nephrotoxicity as a consequence of accumulation has been reported for example for cephalosporin antibiotics (Goldstein, 1993) and antiinflammatory agents (Cox *et al.*, 1990). In our study we used 1) fluorescent substrates and confocal microscopy to study organic anion distribution in intact, isolated PTC and 2) measurements of tracer uptake and respiration is isolated mitochondria to examine underlying mechanisms of uptake. Confocal images of isolated proximal tubular cells showed that the FL analog, CF, exhibited similar properties to the parent compound, and was sequestered mainly within mitochondria. The cytoplasmic fluorescence is low compared to the mitochondrial fluorescence. Because CF (released from CFDA) and FL are distributed similarly, accumulation in mitochondria is a result of intracellular transport properties, rather than a result of concentration of enzymes within mitochondria. The nature and function of a second, vesicular compartment is not yet

TABLE 1

Effect of various anionic drugs on succinate respiration in rat kidney cortex mitochondria

Drug[b]	N	Respiratory Rate (ng atom O/min · mg Prot.)[a]				RCR[d]
		State 2[a]	State 3[a]	State 4[a]	DNP[c]	
Control	9	28 ± 7	97 ± 25	27 ± 6	77 ± 19	3.6 ± 0.2
Fluorescein	7	22 ± 6	67 ± 15*	25 ± 6	64 ± 31	2.8 ± 0.2[e]
p-Aminohippurate	3	17 ± 4	66 ± 8*	24 ± 4	54 ± 18	2.8 ± 0.2[e]
Probenecid	4	26 ± 10	104 ± 10	37 ± 8	93 ± 22	2.8 ± 0.4[e]
Phenylsuccinate	4	32 ± 6	86 ± 14	29 ± 4	63 ± 15	3.0 ± 0.3[e]
Valproate	4	24 ± 6	84 ± 26	31 ± 5	98 ± 6	2.7 ± 0.6[e,f]
Salicylate	4	27 ± 8	90 ± 24	29 ± 10	83 ± 31	3.2 ± 0.4
Indomethacin	4	32 ± 12	87 ± 27	32 ± 11	70 ± 13	2.7 ± 0.6[e]
Naproxen	4	34 ± 10	89 ± 23	35 ± 9	86 ± 22	2.6 ± 0.4[e,f]

[a] Oxygen consumption was measured before ADP addition (state 2), after addition of 0.3 mM ADP (state 3) and after ADP consumption (state 4). All data are expressed as means ± S.D.

[b] Mitochondria were preincubated for 3 min in respiration buffer in presence of 100 μM of the drug tested.

[c] Uncoupled respiratory rate was measured in presence of 44 μM dinitrophenol (DNP).

[d] Respiratory control ratio (RCR) is the ratio of state 3 over state 4 respiration.

[e,f] A one-way analysis of variance followed by the least significant differences test has been used to assess statistical differences between means. [e] $P < .05$ *vs.* control; [f] $P < .05$ *vs.* salicylate.

known. Phenylsuccinate inhibited CF entry into mitochondria, but had no effect on cellular uptake of the CF precursor, CFDA, because CFDA enters cells by simple diffusion and is than hydrolyzed to the fluorescent anion CF.

Phenylsuccinate blocks mitochondrial dicarboxylate uptake irreversibly (Quagliariello *et al.*, 1969; Palmieri *et al.*, 1971). It also blocks CF uptake by mitochondria in intact PTC, suggesting that CF probably enters mitochondria via the dicarboxylate transporter. However, phenylsuccinate seemed to prevent cellular exit of the fluorescent compound as well. As was reported previously, the passive permeability of the brush-border membrane for organic anions is considerably greater than the permeability of the basolateral membrane, and efflux across the brush-border membrane is therefore the principal route of exit (Pritchard and Miller, 1992). Although efflux is energetically downhill and need not be mediated, potential-driven efflux and anion exchange have been reported for organic anions at the brush-border membrane (Russel *et al.*, 1988; Pritchard and Miller, 1992). In addition, Schmitt and Burckhardt (1993) recently found an exchange mechanism for p-aminohippurate with dicarboxylates in bovine brush-border membranes. Although the existence of this exchange mechanism has, as yet, not been shown in rat brush-border membrane, phenylsuccinate might be able to block CF-anion exchange and, as a consequence, CF would be trapped within the proximal tubular cells.

To characterize the interaction between FL and mitochondria, studies were performed with freshly isolated kidney cortex mitochondria, using succinate as substrate. The oxygen uptake results demonstrate that our mitochondrial fraction was of good quality, with oxidation and phosphorylation tightly coupled. The effect of FL and various anionic drugs were tested on succinate stimulated respiration. Indomethacin, naproxen, valproic acid and probenecid tended to increase state 4 respiration. This can be explained as uncoupling of mitochondrial oxidation and phosphorylation, which is in good agreement with earlier findings for these drugs (Pakarinen, 1970; McDougall *et al.*, 1983; Ponchaut and Veitch, 1993). FL, PAH and phenylsuccinate reduced the availability of succinate for oxidation. Dixon analysis revealed that the type of interaction between FL and succinate was competitive. This inhibitory effect may either be a result

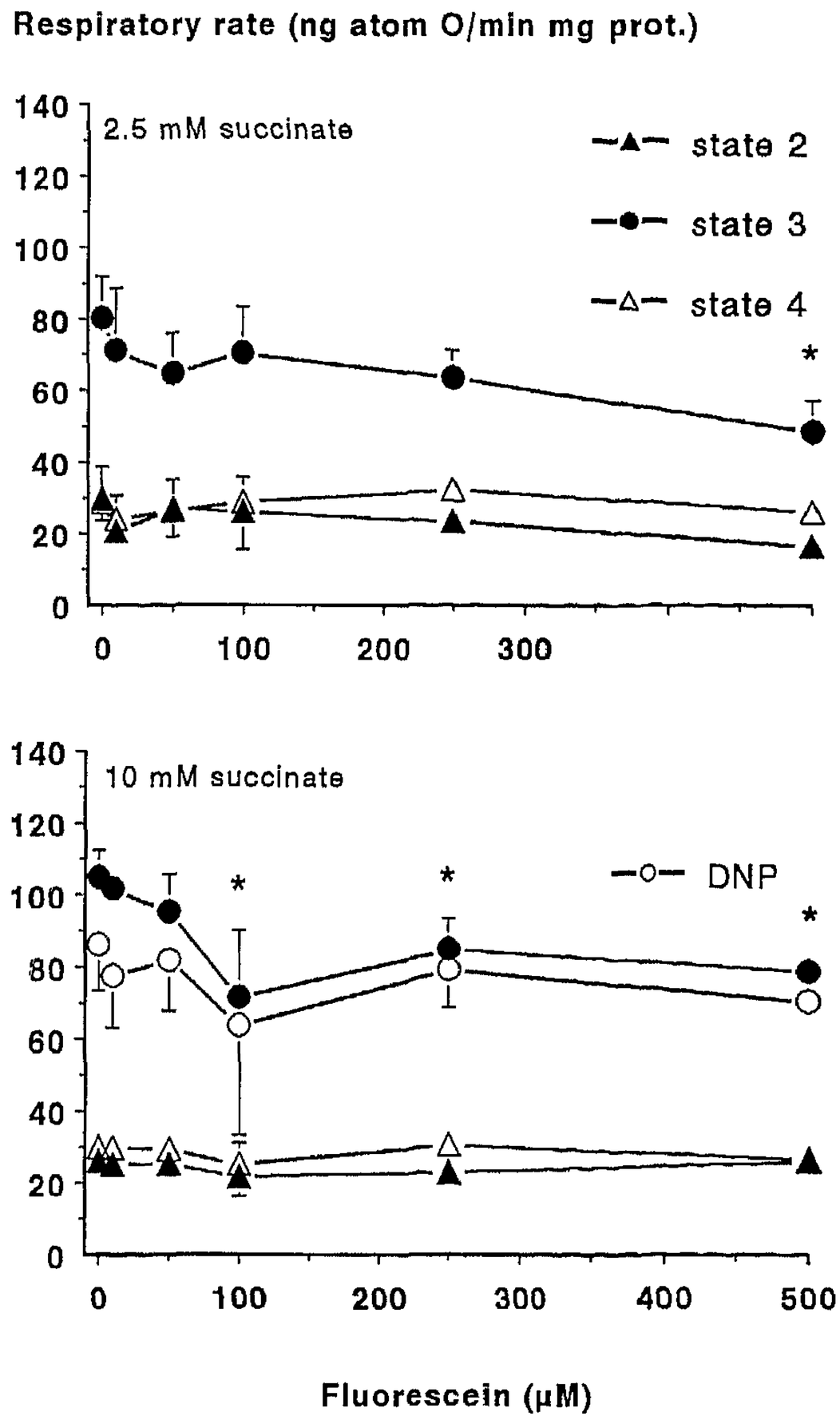


Fig. 4. Upper panel: Respiration of rat kidney cortex mitochondria with 2.5 mM succinate as substrate, after preincubation of mitochondria for 3 min with different concentrations of FL. Respiration was measured in 2 ml buffer at 30°C, in basal state (state 2), in the ADP-stimulated state (state 3) and after ADP consumption (state 4). Data points are expressed as means ± S.D. of three different isolations. Lower panel: Similar conditions as above but 10 mM succinate was used as substrate. DNP: respiratory rate measured after the addition of 44 μM dinitrophenol. Data points are means ± S.D. of four different isolations. *$P < .05$ vs. control respiration (0 μM FL).

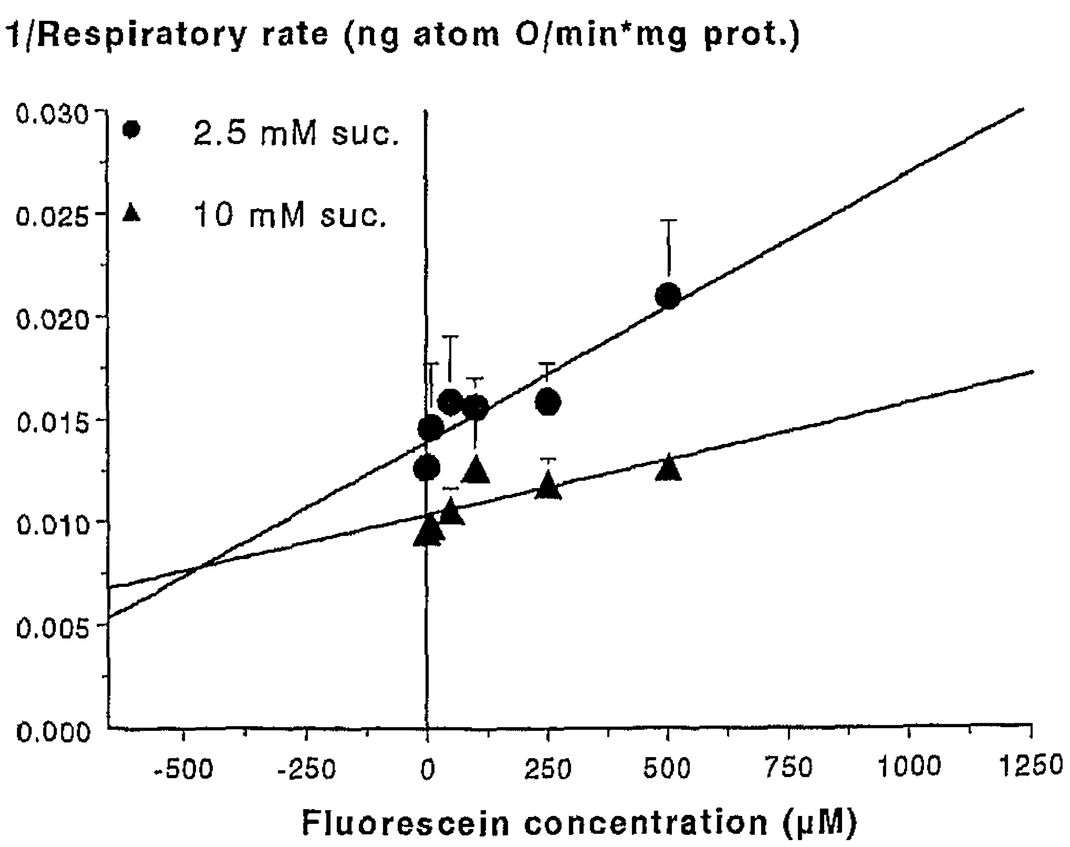


Fig. 5. Dixon-plot of the reciprocal respiratory rate of rat kidney cortex mitochondria, measured after ADP addition (state 3), as a function of FL concentration. Two different substrate concentrations were used (2.5 and 10 mM succinate). Data points are expressed as means of three or four different isolations. The regression equations for both lines are $1.3 \cdot 10^{-5} \cdot \times + 1.4 \cdot 10^{-2}$ ($R^2 = 0.85$) and $5.5 \cdot 10^{-6} \cdot \times + 1.0 \cdot 10^{-2}$ ($R^2 = 0.57$), for 2.5 mM and 10 mM succinate, respectively.

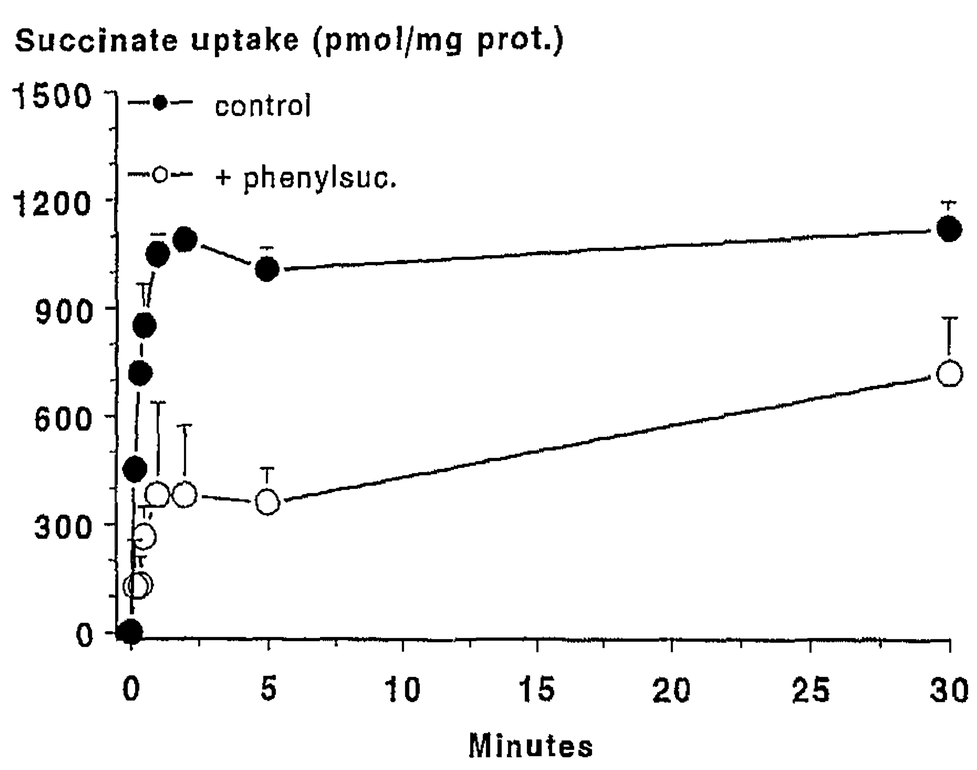


Fig. 6. Time-dependent uptake of ^{14}C-succinate into freshly isolated rat kidney cortex mitochondria at 10°C, with and without preincubation with 12.5 mM phenylsuccinate for 60 min. The extramitochondrial concentration was 500 μM succinate. Phenylsuccinate is the ultimate inhibitor of dicarboxylate-mediated transport in mitochondria and therefore used to determine the contribution of diffusion in transmitochondrial transport of succinate. Values are expressed as means ± S.D. of four different isolations.

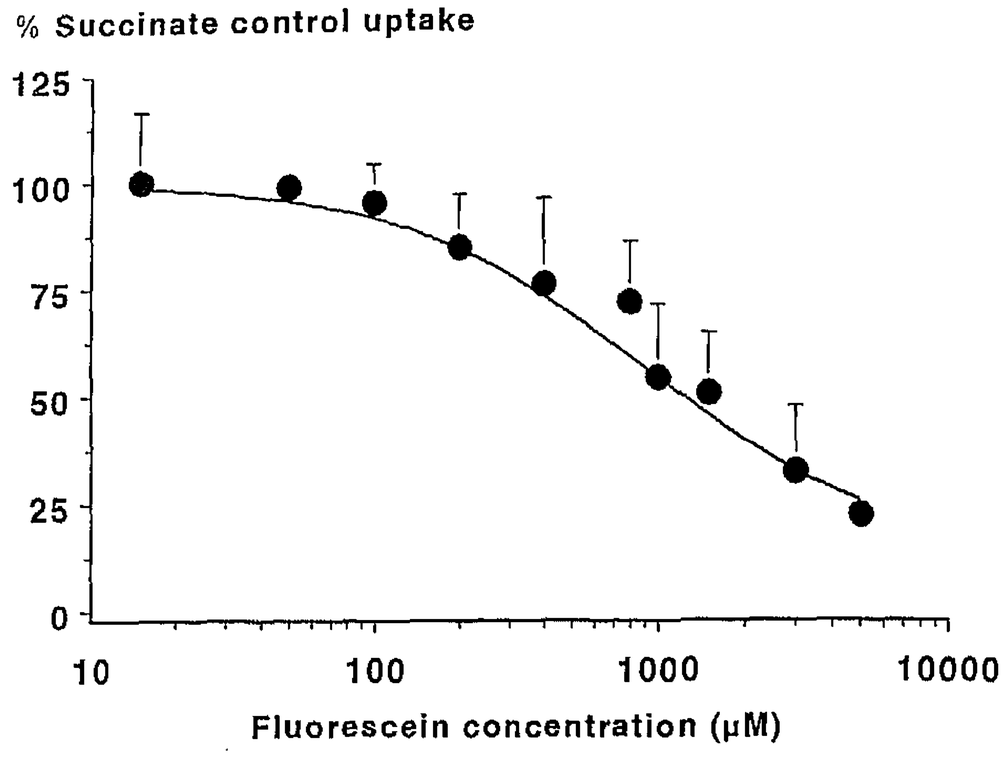


Fig. 7. Inhibition of ^{14}C-succinate uptake into freshly isolated rat kidney cortex mitochondria at 10°C at various concentrations of FL. The extramitochondrial succinate concentration was 100 μM. Inhibition was expressed relative to the maximum inhibition achieved with phenylsuccinate (12.5 mM). Uptake was measured at 10 sec, and values are expressed as means ± S.D. of four different isolations.

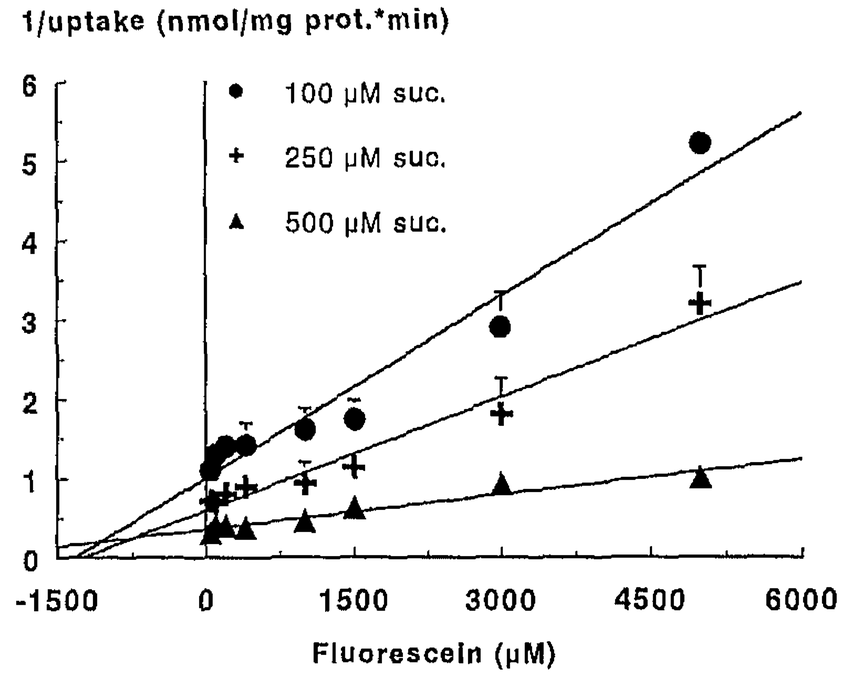


Fig. 8. Dixon-plot of the reciprocal uptake of ^{14}C-succinate into freshly isolated rat kidney cortex mitochondria, as a function of FL concentration. The extramitochondrial concentrations were 100, 250 and 500 μM of succinate. Uptake was measured at 10 sec, and values are expressed as means of four different isolations. The regression equations for the lines are $7.6 \cdot 10^{-4} \cdot \times + 1.01$ (R^2=.95), $4.7 \cdot 10^{-4} \cdot \times + 0.60$ ($R^2 = 0.97$) and $1.5 \cdot 10^{-4} \cdot x + 0.36$ ($R^2 = 0.93$), for 100, 250 and 500 μM succinate, respectively.

of competition for transport across the mitochondrial inner membrane, or competition for binding at the enzyme complex succinate-coenzyme Q dehydrogenase.

To distinguish between these effects, uptake of ^{14}C-labeled succinate was measured in mitochondria with metabolism blocked by rotenone and antimycine. FL inhibited succinate uptake competitively. The inhibitory constant calculated from the tracer uptake experiments (1.1 mM) was two times higher than the constant obtained from the respiration studies (0.5 mM), although the difference was not significant. An explanation may be the difference in experimental conditions. However, it should be noticed that the rather low correlation coefficient of the regression line from 10 mM succinate data points in figure 5 obviously weakens the reliability of the estimated inihibitory constant of FL with respect to mitochondrial respiration.

Inhibition of succinate uptake by FL together with the confocal observations for CF indicate that FL may be a substrate for the mitochondrial dicarboxylate carrier. Moreover, the comparable effect of PAH on mitochondrial respiration suggests that mitochondrial uptake may also be involved in the intracellular compartmentation of this organic anion. However, only direct measurement of (radiolabeled) FL and PAH uptake will give the decisive answers. It has been shown previously that metabolic substrates affected organic anion uptake in renal proximal tubules. *Cis*-inhibition with 1 mM glutarate, adipate or α-ketoglutarate reduced tubular uptake of 10 μM FL, whereas preincubation with 100 μM of these dicarboxylates stimulated cellular uptake (Sullivan and Grantham, 1992). Nikiforov and Ostretsova (1992) suggested that cellular FL uptake was linked to gluconeogenesis; however, the relationship between cellular uptake and oxi-

dative metabolism remained unexplained. The metabolic substrate, pyruvate (5 mM), stimulated oxygen consumption and tissue accumulation of 50 μM FL in kidney cortex slices. Regarding the results of our investigation, it may be speculated that this increase in tissue FL accumulation was due to an enhanced mitochondrial uptake of this organic anion. Exogenous administered pyruvate is rapidly taken up by the cells and sequestered within mitochondria, where it will be carboxylated to malate via the gluconeogenic pathway. Subsequently, malate is transported to cytoplasm via the dicarboxylate carrier, which may occur in exchange for FL. This could explain the enhanced gluconeogenic rate found in the cortex slices exposed to pyruvate and FL.

It seems unlikely that the interaction between FL and succinate will have clinically significant implications. When FL is used as a diagnostic agent, concentrations reached in plasma will be less than 1 μM. From a previous study we can estimate that the final concentration in renal PTC will never exceed 5 μM, because at low extracellular concentrations the compound accumulates in renal cells reaching a 3- to 5-fold concentration intracellulary (Masereeuw *et al.*, 1994). Our results demonstrate that effective inhibition was not found for concentrations below 100 μM.

In conclusion, the interaction between the organic anion FL and mitochondria seems to be specific. Confocal images showed that the FL analog CF accumulates in mitochondria of rat PTC. Mitochondrial accumulation of CF was inhibited by the dicarboxylate uptake blocker, phenylsuccinate. Respiration and radio-tracer uptake studies showed that FL competitively interacts with the mitochondrial dicarboxylate carrier.

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