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Probenecid interferes with renal oxidative metabolism: A potential pitfall in its use as an inhibitor of drug transport

*,[1]Rosalinde Masereeuw, [1]Ard P. van Pelt, [1]Sandra H.G. van Os, [2]Peter H.G.M. Willems, [1]Paul Smits & [1]Frans G.M. Russel

[1]Department of Pharmacology and Toxicology 233 University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands and [2]Department of Biochemistry, University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

1 The anionic drug probenecid has been traditionally used as an inhibitor of renal organic anion transport. More recently the drug was found to inhibit organic cation transport as well, and it is used to retain intracellularly loaded fluorophores. In these investigations it is implicitly assumed that probenecid performs its activity through competition for transport. Here we studied the possibility that probenecid provokes its effect through inhibition of cellular oxidative metabolism.

2 Oxygen consumption was measured in isolated rat kidney cortex mitochondria. At concentrations of 1 mM or higher, probenecid increased the resting state (state 4) and decreased the ADP-stimulated respiration (state 3). A complete loss in respiratory control was observed at 10 mM probenecid.

3 After incubating isolated rat kidney proximal tubular cells (PTC) for 30 min with probenecid a concentration-dependent reduction in ATP content was observed, which was significant at concentrations of 1 mM and higher. Using digital image fluorescence microscopy the membrane potential in PTC was measured with bisoxonol. The mitochondrial effects of probenecid were paralleled by a depolarization of the plasma membrane, immediately after drug addition.

4 All events are likely to be a result of membrane disordering due to the lipophilic character of probenecid, and may explain, at least in part, the various inhibitory effects found for the drug. We recommend to be cautious with applying probenecid in cellular research.

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Abbreviations: BSA, bovine serum albumin; $DiBAC_4$, bisoxonol; DMEM, Dulbecco's modified Eagle's medium; DNP, dinitrophenol; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; FCS, foetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; PTC, proximal tubular cells; RCR, respiratory control ratio

Introduction

The anionic drug probenecid has been traditionally used as an inhibitor of renal organic anion secretion, ever since this compound was found to reduce the rapid urinary secretion of penicillin and other anionic drugs (Beyer *et al*., 1951). At the proximal tubule, a basolateral organic anion transport system (OAT1) mediates renal anionic xenobiotic secretion in conjunction with facilitated diffusion across the luminal membrane into tubular urine. The organic anion which has been studied most extensively is p-aminohippurate. Competition with this compound for secretion, and inhibition of secretion by probenecid, are usually accepted as evidence for a drug to be a substrate for OAT1 (Sekine *et al*., 1997; Sweet *et al*., 1997). Probenecid itself is also a substrate for the renal organic anion system (Weiner *et al*., 1960; Sheikh & Stahl, 1977; Berndt, 1981). Furthermore, probenecid was found to alter the metabolic clearance of anionic drugs by inhibiting glucuronidation (de Miranda *et al*., 1989; Turner & Brouwer, 1997) or by inducing metabolizing enzymes of the mixed function oxidase system (Bammel *et al*., 1991).

Apart from the effect on organic anion clearance, probenecid was found to decrease the *in vivo* renal clearance of organic cations, e.g. cimetidine and famotidine (Gisclon *et al*., 1989; Inotsume *et al*., 1990), and the zwitter ion ciprofloxacin (Jaehde *et al*., 1995), through inhibition of an active component of excretion. This is remarkable, because the chemical structure of probenecid is unrelated to these compounds. Inhibition of organic cation uptake across the basolateral membrane into tubular cells has been described for probenecid (Boom *et al*., 1992; Brändle & Greven, 1992; Boom & Russel, 1993). In isolated luminal membrane vesicles an interaction was also found with the organic cation exchanger present in these membranes (Hsyu *et al*., 1988; Ott *et al*., 1990), and a competitive type of inhibition was assumed.

More recently, probenecid has been widely used in high concentrations (up to 10 mM) as a pharmacological tool to retain intracellularly loaded anionic fluorophores, such as pH indicators (Weinberg *et al*., 1994) and calcium probes (Ruttner *et al*., 1993; Peters *et al*., 1998). In addition to a direct interaction with transport, it may also be possible that the inhibition of dye 'leakage' by probenecid is mediated at a different level. A few studies reported nonspecific effects of probenecid, among which an altered Ca^{2+}-homeostasis (Scheenen *et al*., 1994) and a reduction in tissue oxygen consumption (Choi & Kim, 1992). Scheenen *et al*. (1994) found markedly broadened Ca^{2+} transients in *Xenopus laevis* melanotrope cells in the presence of probenecid and concluded that the drug might increase the gating probabilities of Ca^{2+}-channels or inhibit Ca^{2+}-extrusion mechanisms. Furthermore, Choi & Kim (1992) reported a reduction of tetraethyl

*Author for correspondence; E-mail: R.Masereeuw@farm.kun.nl

ammonium uptake in rabbit kidney cortical slices, which was likely a result of inhibition of tissue metabolism, rather than an interaction with the organic cation transporter. In this preparation, probenecid inhibited the oxygen consumption significantly at concentrations of 3 mM and higher.

The present study addresses the hypothesis that probenecid interferes with cellular oxidative metabolism, which might explain many of the nonspecific effects found for the drug. To this end, we performed respiration studies with renal mitochondria and measured the ATP concentration and membrane potential of intact renal proximal tubular cells (PTC). We demonstrate that probenecid uncouples mitochondrial oxidative phosphorylation, reduces cellular ATP levels and depolarizes the plasma membrane at concentrations of 1 mM or higher. These events may explain, at least in part, the various inhibitory effects described for probenecid. Therefore, we recommend to be cautious with applying high concentrations of probenecid as a pharmacological tool in cell studies.

Methods

Materials

Probenecid, succinate and dinitrophenol were purchased from Aldrich Chemie (Steinheim, Germany). Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) were obtained from Boehringer Mannheim (Mannheim, Germany). Luciferin, luciferase and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma (St. Louis, MO, U.S.A.) and bisoxonol ($DiBAC_4$) from Molecular Probes (Eugene, OR, U.S.A.). Non-essential amino acids were obtained from ICN (Costa Mesa, CA, U.S.A.). Nutrient Mixture Ham's F12 and foetal calf serum (FCS) were from Life Technologies Ltd., BRL (Paisley, U.K.). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.).

Isolation of mitochondria and respiration measurements

The effect of probenecid on mitochondrial respiration was determined in rat isolated kidney cortex mitochondria. Male Wistar-Hannover rats, weighing 230–280 g, were used. Mitochondria were obtained as described previously (Masereeuw *et al*., 1996) and suspended to 5 mg protein ml^{-1} in respiration medium, containing (mM): mannitol 210, KCl 10, KH_2PO_4 10, EGTA 0.5, Tris-HCl 60, at pH 7.4. Oxygen consumption was measured with a Clarke-type platinum electrode, using 1 mg of mitochondrial protein in 2.0 ml of medium. Basal mitochondrial O_2 consumption was measured at 30°C in respiration medium in the absence of ADP (state 2), in the presence of 0.3 mM ADP (state 3), after ADP consumption (state 4), and after the addition of dinitrophenol (DNP; final concentration 44 μM). Succinate (10 mM) was used as the metabolic substrate, and rotenone (1 μM) was added to block electron transport proximal to succinate entry into the respiratory chain. Respiratory rates were calculated and expressed as ng atoms of oxygen per min per milligram of protein (ng atom O min^{-1} mg prot.$^{-1}$).

Isolation of rat PTC and cellular ATP determination

Rat kidney proximal tubular cells (PTC) were isolated from male Wistar-Hannover rats (230–280 g) as described previously (Masereeuw *et al*., 1994) and suspended to 10–15 mg protein ml^{-1} in incubation buffer containing (mM): NaCl 117.5, KCl 4, $MgSO_4$ 1.2, KH_2PO_4 0.95, $NaHCO_3$ 22.5, glucose 11.1, and $CaCl_2$ 2.5. ATP content in proximal tubular cells was determined by a modification of Miller & Horowitz (1986) of the luciferin-luciferase procedure. Briefly, an aliquot of 1–1.5 mg cell protein was incubated in 500 μl buffer alone (control) or supplemented with various inhibitors, and incubation took place for 30 min at 37°C under an atmosphere of 95% oxygen and 5% carbon dioxide. Subsequently, the samples were centrifuged for 3 min at 100 $\times g$ and 4°C, and 100 μl 3 N perchloric acid was added to the pellet, which was then vortex-mixed, let 5 min to rest, again vortexed and centrifuged for 3 min at 100 $\times g$. The sample was neutralized with 125 μl medium containing 2 N KOH, 0.4 M imidazole and 0.4 M KCl, vortex-mixed and centrifuged again. The supernatant was diluted 25 times and to 50 μl of this solution 500 μl assay medium was added, containing 50 mM glycylglycine, 7.5 mM dithiothreitol, 2 mM EGTA, 2 mM $MgCl_2$, 0.04% (w v^{-1}) BSA, 10 μg ml^{-1} luciferin, and 2000 U ml^{-1} luciferase at pH 8.0, and measured after 30 min by using a liquid scintillation counter. ATP concentrations were calculated by comparing scintillation counts with a calibration curve of various concentrations of ATP in perchloric acid : neutralization buffer 1 : 1.25. Protein content in proximal tubular cells and mitochondrial fraction was determined using the BioRad Protein Assay of BioRad (München, Germany) with BSA as the protein standard.

Digital image fluorescence microscopy

Rat kidney PTC were isolated as described above and cultured for 1 day in DMEM/Ham's F12 (1 : 1, v v^{-1}) medium supplemented with 10% (v v^{-1}) FCS, 5 pM triiodothyrnin, 5 μg ml^{-1} transferrin, 0.1 IU ml^{-1} insulin, 50 nM hydrocortison, 5 ng ml^{-1} prostaglandin E1, 50 nM Na_2SeO_3, 1.9 mM glutamine and 10 μg ml^{-1} gentamicin. Cells were plated on rat tail collagen-coated glass coverslips and transferred to 6-well culture dishes (Costar, Cambridge, MA, U.S.A.) filled with 2 ml of culture medium. The next day, cells were washed in incubation buffer and placed in a chamber with a volume of 1 ml and incubated with fluorescent dye. For membrane potential experiments, 750 nM of bisoxonol ($DiBAC_4$) was present in the medium. Dynamic video imaging was carried out using the MagiCal hardware and Tardis software of Joyce Loebl as described previously (Tyne and Wear, U.K.; de Roos *et al*., 1997). Loaded cells were excited with 490 nm and emitted light was filtered through a 510 nm filter and captured by a CCD camera. Neutral density filter of 1.5 was used to diminish bleaching for bisoxonol.

Data analysis

All data are expressed as mean $\pm$ s.d. Statistical differences between means were determined with Student's *t*-test. In respiration measurements, the multiple means were compared by using one-way analysis of variance followed by the least significant difference *post hoc* test. The level of significance was set to $P<0.05$. The log-concentration response curves were analysed by means of nonlinear regression with the program GraphPad Prism™ (version 3.00 for Windows; GraphPad Software, San Diego CA, U.S.A.), assuming a one site competition model. All curves were fitted using individual data. In membrane potential measurements the depolarization by probenecid was expressed relative to the maximum depolarization achieved with sodium azide (0.5 mM), and determined according to:

$$\text{Effect} = \frac{E_{\text{probenecid}} - E_0}{E_{\text{sodium azide}} - E_0} \quad (1)$$

Where E_0 is the baseline fluorescence measurements, $E_{\text{probenecid}}$ is the fluorescence determined after addition of probenecid and $E_{\text{sodium azide}}$ after addition of sodium azide.

Results

Effect of probenecid on mitochondrial respiration

The effect of probenecid on the different respiration states in isolated rat kidney cortex mitochondria was assessed within the concentration range of 0.1–10 mM (six different concentrations). Energization of the mitochondria was achieved by the addition of 10 mM succinate. Subsequently, the oxygen consumption was measured (Table 1) prior to ADP addition (state 2), after adding ADP (state 3) and after ADP consumption (state 4). Uncoupled respiratory rate was measured in the presence of 44 μM DNP. The quality of the mitochondrial preparation was assessed by the respiratory control ratio (RCR), which is the ratio of state 3 over state 4 respiration and gives an indication of the coupling between oxidation and phosphorylation. Energized mitochondria showed an RCR of 3.6 ± 0.4 ($n=6$), indicating that our preparation was of good quality and tightly coupled. Probenecid induced a concentration-dependent decrease in state 3 and increase in states 2 and 4 respiration (Figure 1), which became significant at 1 mM. At 10 mM a complete loss of respiratory control is observed. Consequently, a reduction in RCR is observed and a plot of the log-concentration probenecid versus RCR resulted in a sigmoidal curve (Figure 2 upper panel). This curve was described best according to a one-site competition model. The concentration of inhibitor causing 50% inhibition of RCR (IC_{50}), was 0.9 ± 0.3 mM (Table 2). Figure 2 (lower panel) shows that a similar relationship was found for probenecid on the uncoupled respiratory rate after the addition of DNP. This uncoupler discharges the proton electrochemical gradient across the mitochondrial inner membrane, thereby stimulating oxygen consumption without ATP synthesis. An IC_{50} value of 2.6 ± 0.2 mM could be determined for the concentration-dependent effect of probenecid on DNP-uncoupled respiratory rate (Table 2).

Effect of probenecid on cellular ATP content

A reduction in uncoupled respiration is an indication of loss of respiratory control and a decrease in mitochondrial oxidative metabolism. This may lead to a reduction in cellular ATP concentration. By means of the luciferin-luciferase method an intracellular ATP concentration of 3.1 ± 0.7 nmol mg prot^{-1} was determined in control PTC, incubated for 30 min without further additions. When PTC were incubated for 30 min with

Table 1 Oxygen consumption in energized rat kidney cortex mitochondria[a]

Condition	*Respiratory rate* (ng atom O min^{-1} mg prot.$^{-1}$)
State 2	30 ± 6
State 3	104 ± 14
State 4	29 ± 4
Uncoupled	111 ± 17

[a]Oxygen consumption was measured prior to ADP addition (state 2), after addition of 0.3 mM ADP (state 3) and after ADP consumption (state 4). [b]Uncoupled respiratory rate was measured in presence of 44 μM DNP. All data are expressed as means $\pm$ s.d. ($n=6$).

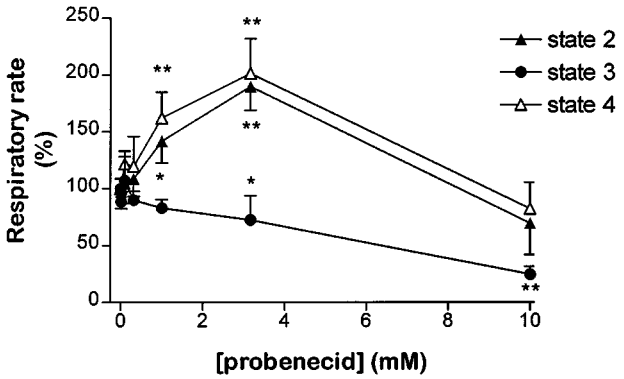

Figure 1 Effect of probenecid on succinate-stimulated respiration. Oxygen consumption in the different respiration states was measured using rat kidney cortex mitochondria, prior to ADP addition (state 2), after adding ADP (state 3) and after ADP consumption (state 4), for control mitochondria and in presence of various concentrations of probenecid. Data are expressed as per cent of control respiratory rate and presented as means $\pm$ s.d. for 3–4 different isolations. Statistical comparisons: significantly different from control situation, $*P<0.05$, $**P<0.01$.

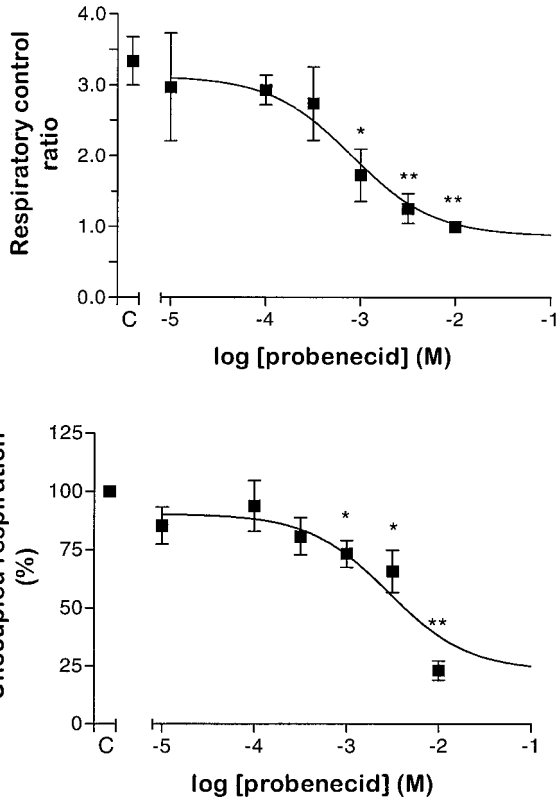

Figure 2 Upper panel: Concentration-dependent reduction of mitochondrial respiration by probenecid. Respiratory control ratio is the ratio of state 3 over state 4 respiration, determined after addition of various concentrations probenecid. Lower panel: Effect of probenecid on uncoupled respiration. Uncoupled respiratory rate was measured after the addition of 44 μM DNP in presence of various concentrations probenecid. Data are expressed as per cent of control and presented as means $\pm$ s.d. for 3–4 different isolations. C: control value (in absence of probenecid). Statistical comparisons: significantly different from control situation, $*P<0.05$, $**P<0.01$.

various concentrations of probenecid a clear concentration-dependent loss in ATP content was observed, which was significant at 1 mM and higher concentrations of the drug (Figure 3). At 10 mM probenecid, the cellular ATP concentration was reduced to 26±7% of control ATP. After nonlinear regression analysis, an IC_{50} value of 2.1±0.3 mM could be calculated, which is comparable with the IC_{50} values determined for the effects of probenecid on mitochondrial respiration (Table 2). Moreover, the inhibitory potencies are in good agreement with the IC_{50} value of 0.7 mM determined for probenecid on the uptake of the cationic drug cimetidine, in the same preparation (Boom & Russel, 1993).

Effect of probenecid on membrane potential

The effect of probenecid on cellular integrity was investigated by measuring the membrane potential. Figure 4 shows a representative comparison of membrane potential measurements in control situation and after treatment with 3.2 mM probenecid. Directly after addition of probenecid a depolarization of the membrane is observed, again in a concentration-dependent manner. Figure 5 shows the log concentration-depolarization plot obtained after non-linear regression analysis. An IC_{50} value of 0.30±0.02 mM was obtained, which is in the same range as the inhibitory potencies of probenecid on mitochondrial respiration and cellular ATP content (Table 2).

Table 2 Inhibitory potencies of probenecid analysed by different parameters[a]

Preparation	*Experimental condition*	$-\log IC_{50}$
Mitochondria	Respiratory Control Ratio	3.07±0.15
Mitochondria	Uncoupled respiration	2.59±0.19
PTC	Cellular ATP content	2.71±0.11
PTC	Membrane depolarization	3.52±0.28
PTC[b]	Cimetidine uptake	3.15±0.31

[a]Curves were analysed using GraphPad Prism™. The $-\log IC_{50}$-value is the negative logarithm of the molar concentration required to produce half-maximum inhibition. [b]Adapted from (Boom & Russel, 1993). Data are expressed as means±s.d. ($n=3-7$).

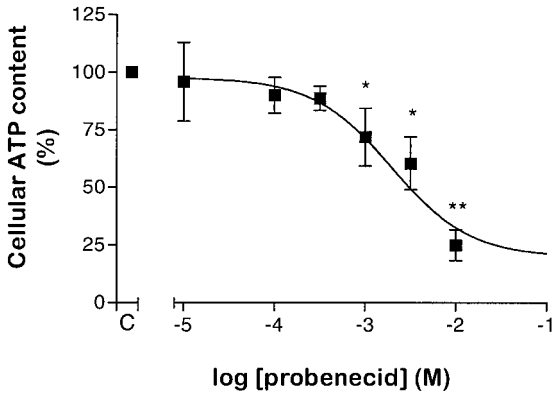

Figure 3 Effect of probenecid on cellular ATP content. For ATP measurements PTC were incubated for 30 min with various concentrations of probenecid, after which ATP concentrations were determined as described in Methods. Data are expressed as per cent of control and presented as means±s.d. of 3–4 different isolations. C: control value (in absence of probenecid). Statistical comparisons: significantly different from control situation, $*P<0.05$, $**P<0.01$.

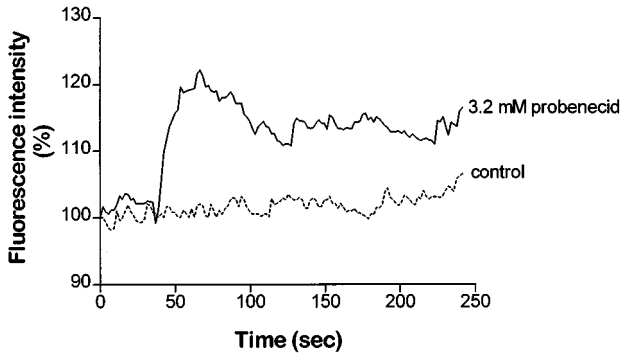

Figure 4 Representative trace of membrane potential measurements in renal PTC. Cells were loaded with the probe bisoxonol (750 nM) and relative fluorescence was determined in controls (no additions) and after adding 3.2 mM probenecid, as described in Methods. Lines are means of 3–8 cells from one isolation.

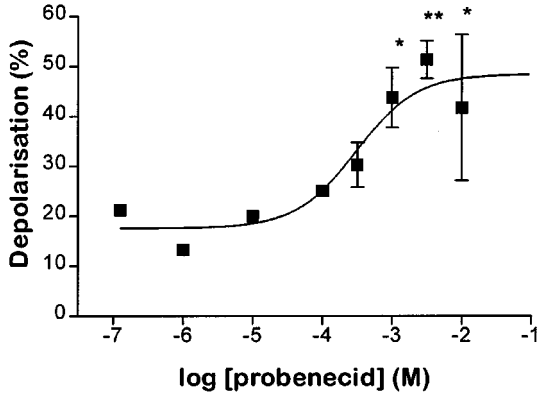

Figure 5 Concentration-dependent depolarization of the plasma membrane induced by probenecid. Cells were loaded with the probe bisoxonol (750 nM), and relative fluorescence was measured. The depolarization by probenecid was determined as described in Methods and equation 1. Data are expressed as per cent of sodium-azide-dependent depolarization and presented as means±s.d. of 3–12 cells of three different isolations. Statistical comparisons: significantly different from control situation, $*P<0.05$, $**P<0.01$.

Discussion

Renal proximal tubule cells serve an important role in the extrusion of endogenous and exogenous compounds from blood into urine. These cells have a high metabolic demand and are equipped with specific secretory proteins that remove the substrates (Pritchard & Miller, 1993; Ullrich, 1997; Roch-Ramel, 1998). Proximal tubule cells rely almost exclusively on oxidative phosphorylation for ATP synthesis (Gullans & Mandel, 1992), and, therefore, may be particularly vulnerable to mitochondrial toxicity. The mitochondrial respiratory chain in the inner membrane is proton translocating, hereby creating an electrochemical proton gradient across the membrane. In general, lipid-soluble weak acids can act as proton carriers and provide a pathway for the flow of protons across the inner membrane in addition to the ATP synthase. As a result, no proton gradient is generated and ATP can no longer be produced. This causes an increase in oxidation of substrate, as reflected by an enhanced control respiratory rate (state 4), resulting in a decrease in respiratory control ratio (Terada, 1990). Probenecid is an acidic drug, and the results of the present study indicate that it acts as an uncoupler of oxidative phosphorylation. Simultaneously, the drug reduced state 3 respiration. At higher concentrations probenecid reduced the

uncoupled respiratory rate in a concentration-dependent manner, indicating inhibition of the respiratory chain. Unlike DNP which is a pure protonophore, probenecid may thus be classified as a structural uncoupler impairing all steps of oxidative phosphorylation, as is known for phospholipases, detergents and mechanical disruptors (Hanstein, 1976).

Probenecid was shown to be an efficient inhibitor of the renal secretion of organic anions and cations, but an important difference between these interactions is the inhibitory potency by which the effects are exerted. Apparent inhibitory constants ranging between 10–100 μM were reported against the uptake of penicillin or PAH, using kidney cortex slices (Nierenberg, 1987) or isolated renal membrane vesicles (Russel *et al*., 1988), whereas the inhibitory effect of probenecid on organic cation transport was at least 10 fold lower in isolated renal membrane vesicles (Hsyu *et al*., 1988) or PTC (Boom & Russel, 1993). The present study describes the effects of probenecid on mitochondrial respiratory control, uncoupled respiratory rate as assessed by DNP addition, and cellular ATP content. The apparent inhibitory constants determined are all in the same order of magnitude, and not significantly different from the inhibitory constant reported for probenecid on cimetidine uptake in PTC (Boom & Russel, 1993). The competitive interaction between probenecid and organic anion uptake in renal proximal tubules is most likely not influenced by the effects on cell metabolism at low concentrations. However, our present findings suggest that the inhibition of probenecid on the organic cation, cimetidine, uptake is nonspecific and probably due to the influences on cellular oxidative metabolism.

Organic cation entry depends primarily on transmembrane electrical potential difference (Pritchard & Miller, 1997; Koepsell *et al*., 1999), and modulating the membrane potential should affect organic cation uptake. It was shown previously that depletion of ATP initiates a loss in the strength of membrane potential retention (Doctor *et al*., 1997). We, therefore, investigated whether a depolarization of the plasma membrane results from probenecid treatment. Our findings with digital image fluorescence microscopy are consistent with a depolarization of the basolateral membrane. The potency at which probenecid affects the membrane potential lies in the same range as its inhibitory potency on other cellular events, suggesting that the inhibitory effect on organic cation transport in PTC may be explained by a depolarization of the basolateral membrane. Furthermore, our results are in close agreement with the results of Smith *et al*. (1988), who showed that uptake of the cationic drug tetraethylammonium in teleost proximal tubules was inhibited by basolateral membrane depolarization and stimulated by hyperpolarization.

It is remarkable that the probenecid-induced membrane depolarization occurred immediately after addition. The membrane potential of the proximal tubule cell is maintained by Na^+-K^+-ATPase activity, but inactivation of this pump results in just a slight drop in membrane potential. On the other hand, a small change in ion flow across the cell membrane may result in a significant change in membrane potential. Therefore, it may be speculated that cell membrane depolarization is not a result of ATP depletion, but rather reflects interference of probenecid with membrane permeability. Similar findings have been reported for the nonsteroidal anti-inflammatory drugs salicylate (metabolite of aspirin), diclofenac and diflunisal on mitochondrial membrane potential (McDougall *et al*., 1983; Petrescu & Tarba, 1997). These drugs were found to be uncouplers of oxidative phosphorylation as well, and it was suggested that the mechanism of mitochondrial membrane depolarization is either a result of membrane disordering due to the lipophilic character of the drugs (Uyemura *et al*., 1997) or, more specific, through a decrease in protonmotive force due to proton translocation (Gutknecht, 1992). In both cases, the mitochondrial membrane permeability for protons is altered. The finding that probenecid not only affects state 4 respiration, but inhibits state 3 in mitochondria as well, is consistent with a nonspecific effect on membrane permeability. Considering the hydrophobic properties of the drug, it is to be expected that probenecid accumulates in the membranes of the cell. Further research is needed to resolve this issue.

Probenecid has been used as a pharmacological tool in many different experimental settings next to renal transport inhibition, e.g. in measurements of intracellular pH (Weinberg *et al*., 1994) and free Ca^{2+} (Ruttner *et al*., 1993; Peters *et al*., 1998), and cell volume control (Kanli & Terreros, 1997). In the first two experimental conditions probenecid was used in millimolar concentrations to retain intracellularly loaded fluorophores. From our findings it may be clear that one should be cautious with interpreting the results obtained in these studies, because probenecid likely disturbs cellular homeostasis under these conditions. A similar conclusion was drawn by Scheenen *et al*. (1994), who observed a markedly altered Ca^{2+} homeostasis in single melanotrope cells of *Xenopus laevis*, and Choi & Kim (1992), who found a reduction in tissue oxygen consumption after addition of probenecid. However, both groups did not have a clear explanation for their observations.

In summary, our data indicate that probenecid uncouples mitochondrial oxidative phosphorylation, reduces cellular ATP levels and depolarizes the plasma membrane. These events are likely to be a result of membrane disordering due to the lipophilic character of probenecid, and may explain, at least in part, the various inhibitory effects found for the drug. Therefore, we recommend to be cautious with applying high concentrations of probenecid as a pharmacological tool in cellular research.

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