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PHLORETIN – AN UNCOUPLER AND AN INHIBITOR OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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The effect of phloretin on respiration by isolated mitochondria and submitochondrial particles was studied. In submitochondrial particles, both NADH- and succinate-dependent respiration was inhibited by phloretin. 50% maximum inhibition was reached at phloretin concentrations of 0.1 mM (NADH oxidation) and 0.7 mM (succinate oxidation). In isolated mitochondria, phloretin inhibited glutamate oxidation in both State 3 and State 4; 50% maximum inhibition occurred at about 30 μM. Succinate oxidation is inhibited in State 3 by phloretin, inhibition being half its maximum value at 0.5 mM, but in State 4 it is stimulated about 2-fold by phloretin at a concentration of 0.6 mM. Ascorbate oxidation is stimulated in both State 3 and State 4, maximum stimulation being equal to that obtained with an uncoupler of oxidative phosphorylation. Under all circumstances, phloretin lowered the transmembrane electrical potential difference in isolated mitochondria. These results are discussed in terms of mosaic non-equilibrium thermodynamics. We conclude that phloretin is both an uncoupler and an inhibitor of oxidative phosphorylation.

Introduction

Inhibitors of membrane glucose transport, such as cytochalasin B, phloretin and phlorizin, are frequently used to study transport kinetics in fat cells with a small intracellular water space which is rapidly saturated. For the investigation of non-equilibrium kinetics, short incubation periods are essential [1]. We used 2-deoxyglucose as a marker of glucose uptake in rat adipocytes. It is presumed that after translocation 2-deoxyglucose is phosphorylated but not further metabolized. The rate of accumulation of 2-deoxyglucose reflects the transport process, assuming that the phosphorylation reaction is not rate limiting and 2-deoxyglucose phosphate is not rapidly dephosphorylated in the cell [2]. However, we found that the accumulation of 2-deoxyglucose fluctuates [3]. This suggests that 2-deoxyglucose phosphate can be rapidly dephosphorylated and leave the cell. Further studies revealed that inhibitors and uncouplers of oxidative phosphorylation, such as KCN and 2,4-dinitrophenol, also induce rapid dephosphorylation of intracellular 2-deoxyglucose phosphate. Phloretin, phlorizin and cytochalasin B could not prevent the release of 2-deoxyglucose. Moreover, in con-

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

trast to the other inhibitors, phloretin caused rapid dephosphorylation of 2-deoxyglucose phosphate [4]. A simultaneous rapid decline in ATP levels was also observed. The similarity between the effects of phloretin and KCN or 2,4-dinitrophenol could reflect a direct effect of phloretin on mitochondria. The effect of phloretin on isolated rat liver mitochondria was investigated by Kimura and co-workers [5], but they were unable to explain the mechanism of the phenomenon on the basis of their results. Therefore, we performed additional experiments using rat liver mitochondria and bovine heart submitochondrial particles. These experiments led us to the conclusion that phloretin has both an uncoupling and an inhibitory effect on mitochondrial oxidative phosphorylation.

Methods and Materials

Rat liver mitochondria were isolated as described by Hogeboom [6] and modified by Myers and Slater [7], resuspended at a concentration of 20 mg protein/ml in 0.25 M sucrose, stored on crushed ice and used within 5 h after isolation. Mg/Mn/ATP/succinate submitochondrial particles were isolated from heavy bovine heart mitochondria as described by Hansen and Smith [8] and modified by De Jonge and Westerhoff [9], stored at 253 K for at the most 14 days in small aliquots and used within 24 h after thawing.

Oxygen uptake was measured in a thermostatically controlled 1.5 ml oxygraph vessel containing a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) filled with 5 M choline chloride and a potassium-sensitive combination electrode (type 10405 3754; Ingold, Urdorf, Switzerland). Protein was determined by the biuret method. Phloretin and phlorizin were obtained from K&K, Plainview, NY, U.S.A. Stock solutions of phloretin and phlorizin at a concentration of 0.3 M in ethanol were stored for at the most 14 days at 253 K. Phlorizin was hydrolyzed according to the method of Müller and Robertson [10]; the hydrolysate was washed three times with ice-cold water, dissolved in an excess of ice-cold ethanol and filtered. The filtrate was evaporated in a vacuum and the crystals dissolved in 0.5 ml of ethanol, giving a concentration of less than 0.5 M.

All reagents were of the highest purity commercially available. Deionized, Millipore-filtered water was used throughout the experiments.

Results

The effects of phloretin on the rate of respiration of rat liver mitochondria are shown in Fig. 1. A strong inhibition of glutamate-dependent respiration was observed in both State 3 (in the presence of 3 mM ADP) and State 4. The stimulatory effect of ADP was abolished at phloretin concentrations of 40 μM or more. At 200 μM respiration was less than 20% of the original (State 4) rate. In contrast, phloretin concentrations of up to 1 mM stimulated succinate oxidation in State 4 but inhibited respiration in State 3 (Fig. 1B). The concentration of phloretin necessary for the inhibition of glutamate-dependent respiration was an order of magnitude lower than that required for succinate-dependent respiration.

To investigate whether phloretin gives rise to a highly localized block in the respiratory chain, we also examined the effect of phloretin on mitochondria that oxidize ascorbate plus TMPD as a mediator (Table I). In both State 3 (Expt. 2) and State 4 (Expts. 1 and 3) we observed a marked stimulation of ascorbate oxidation by phloretin. This stimulation was equal to the stimulation obtained with an excess of FCCP (Expts. 2 and 4).

The experiments with succinate and glutamate were all carried out in the presence of valinomycin which made it possible to monitor (with a K^+-sensitive electrode) the changes in extramitochondrial K^+ concentrations. Assuming electrochemical equilibrium for K^+, the electrical potential gradient across the mitochondrial inner membrane, $\Delta\psi$, can be calculated from the formula [11]:

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K^+]_{in}}{[K^+]_{out}} = 61.5 \log \frac{[K^+]_{in}}{[K^+]_{out}} \quad \text{(in mV)} \qquad (1)$$

where:

$$\frac{[K^+]_{in}}{[K^+]_{out}} = \frac{S-1}{V_R} + 1 \qquad (2)$$

$$V_R = \frac{\text{total mitochondrial inner volume}}{\text{total outer volume}},$$

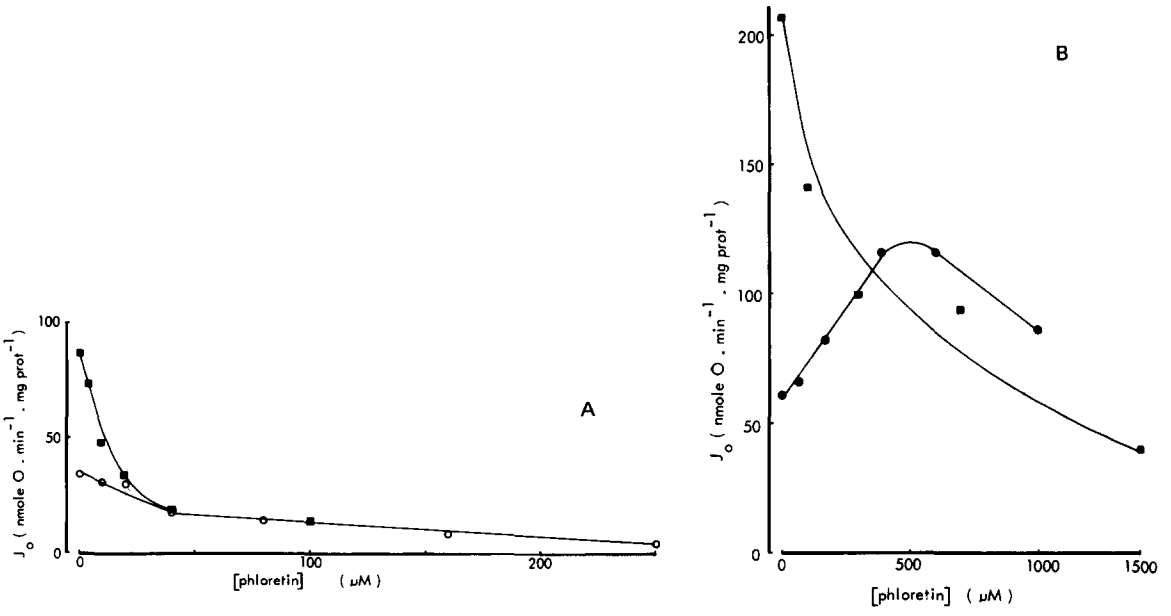

Fig. 1. Effect of different phloretin concentrations on glutamate (A) and succinate (B) oxidation in isolated mitochondria. Rat liver mitochondria (1.2 mg protein) were incubated at 310 K in 1.4 ml of a medium containing 250 mM sucrose, 10 mM phosphate buffer (brought to pH 7.3 with Tris base) 5 mM $MgCl_2$, 0.5 mM EGTA and 0.4 mg/l valinomycin. 3 mM Tris-ADP was added (■) or omitted (○, ●). Phloretin was added in amounts, listed under the abscissa, as a 0.3 M or, when the final concentration was less than 100 μM, as a 30 mM solution in absolute ethanol. Further additions were 3.5 mM Tris-glutamate plus 2 mM Tris-malate (A) or 10 mM Tris-succinate plus 0.7 mg/l rotenone (B).

TABLE I

EFFECT OF PHLORETIN AND FCCP ON THE RATE OF MITOCHONDRIAL ASCORBATE OXIDATION

Rat liver mitochondria (2 mg protein) were incubated at 310 K in 1.4 ml of a medium containing 250 mM sucrose, 10 mM phosphate buffer (brought to pH 7.3 with Tris base), 5 mM $MgCl_2$, 5 mM KCl, 0.5 mM EGTA and 5 mM Tris-ascorbate. Tris-ADP was added as a 100 mM aqueous solution (pH about 7.5), and TMPD was added as a 10 mM aqueous solution. Phloretin was added as a 0.5 M solution in absolute ethanol.

Expt. No.	[TMPD] (μM)	[ADP] (mM)	[FCCP] (mg/l)	[Phloretin] (mM)	Respiration rate (nmol O/min per mg protein)
1	50	–	–	–	14
	50	–	–	1	32
	50	–	–	3	27
	50	–	–	6	21
2	50	–	–	–	16
	50	2	–	–	23
	50	2	–	1	30
	50	2	–	3	26
	50	2	1.5	3	25
3	1250	–	–	–	78
	1250	–	–	1	230
4	1250	–	–	–	70
	1250	–	1.5	–	220

$$S = [K^+]_{out}^{de\text{-}energized} / [K^+]_{out}^{energized}$$

and $[K^+]_{out}^{de\text{-}energized}$ is the outside K^+ concentration after addition of an excess amount of FCCP. Since, in the presence of 10 mM phosphate, changes in the transmembrane pH gradient (ΔpH) are negligible [12], changes in $\Delta\psi$ will reflect changes in the total transmembrane proton-motive force $\Delta\tilde{\mu}_{H^+}$. After calibrating the K^+ electrode with standard KCl solutions and assuming an internal mitochondrial water space of 1.5 μl/mg protein [13], we calculated the electrical potential gradient for different phloretin concentrations (Table II). Since we neglected energy-dependent swelling, these values must be considered as lower limits. From the results obtained when succinate was the substrate (Expt. 1 in Table II), we concluded that phloretin does not depress the proton-motive force by decreasing respiration (i.e., the rate of proton pumping) but primarily by increasing the proton permeability of the membrane: addition of phloretin makes the respiration increase and the membrane potential decrease. Of course, Fig. 1B reminds us that there is also an inhibitory effect on respiration.

The greater inhibition by phloretin of glutamate oxidation, compared to succinate oxidation (Fig. 1), could be due to an effect of phloretin on a system other than the respiratory chain, e.g., transamination, or a specific effect on NADH:Q oxidoreductase. The decrease in proton-motive force caused by phloretin could lower the flux through the (electrogenic [14]) glutamate/aspartate antiporter to such an extent that this carrier becomes rate limiting. However, this possibility was excluded by Expts. 4 and 5 (Table I) which show that, at concentrations that gave a similar reduction in proton-motive force, phloretin and FCCP decreased and increased, respectively, the rate of glutamate-dependent respiration by rat liver mitochondria. To explore these effects further, we carried out experiments with submitochondrial

TABLE II

EFFECT OF PHLORETIN AND FCCP ON RESPIRATION RATE AND MEMBRANE POTENTIAL OF RAT LIVER MITOCHONDRIA

Mitochondria (1.2 mg protein) were incubated at 310 K in 1.4 ml of a medium containing 250 mM sucrose, 10 mM phosphate buffer (brought to pH 7.3 with Tris base), 5 mM $MgCl_2$, 0.5 mM EGTA and 0.4 mg/l valinomycin. 3 mM Tris-ADP was added as a 100 mM aqueous solution. Phloretin was added as a 0.3 M or, when final concentration was less than 100 μM, as a 30 mM solution in absolute ethanol. Further additions were 3.5 mM Tris-glutamate plus 2 mM Tris-malate (glutamate as substrate) or 10 mM Tris-succinate plus 0.7 mg/l rotenone (succinate as substrate).

Expt. No.	Substrate	[ADP] (mM)	[FCCP] (mg/l)	[Phloretin] (μM)	$\Delta\psi$ (mV)	Respiration rate (nmol O/min per mg protein)
1	succinate	–	–	–	185	62
		–	–	100	177	79
		–	–	300	155	116
		–	–	700	120	109
2	glutamate	–	–	–	175	21
		3	–	–	156	66
		3	–	40	141	7
		3	–	130	120	0
3	glutamate	–	–	–	177	29
		3	–	–	157	92
		3	1	–	153	85
		3	3	–	141	42
4	glutamate	–	–	–	171	34
		–	–	10	166	31
5	glutamate	–	–	–	177	34
		–	1	–	166	55

particles, which are oriented inside-out compared to mitochondria. We did not see any effect of phloretin at concentrations of up to 3 mM on the oxidation of ascorbate plus TMPD. The effects of phloretin on succinate- and NADH-dependent respiration are shown in Fig. 2. Because respiration of the submitochondrial particles could not be stimulated by ADP, we used FCCP to obtain 'State 3-like' oxidation.

Again, NADH oxidation was much more sensitive to inhibition by phloretin than succinate oxidation. In submitochondrial particles, neither NADH nor succinate oxidation was stimulated by phloretin (succinate oxidation was also barely stimulated by FCCP). We concluded that substrate and/or matrix reactions could not account for the differences in susceptibility towards inhibition by phloretin between NADH and succinate oxidation.

Similar experiments were carried out with the glycoside form of phloretin, phlorizin. In concentrations of up to 3 mM, this compound had no effect on respiration by mitochondria and submitochondrial particles. To determine whether the effects of phloretin were due to contamination, we hydrolyzed some phlorizin (see Methods and Materials) and examined the effect of the hydrolysate. Since we indeed found inhibition of the respiration at the expected concentrations in both mitochondria and submitochondrial particles, we concluded that phloretin and not a contaminant gave the reported results.

Discussion

The experiments described above show that phloretin inhibits glutamate and succinate but not ascorbate oxidation in mitochondria in State 3 and inhibits glutamate but stimulates succinate and ascorbate oxidation in State 4. This latter finding could be explained by an uncoupling in addition to an inhibiting effect, where glutamate oxidation is especially sensitive to inhibition by phloretin due to an effect on the NADH : Q oxidoreductase segment, transamination or the electrogenic [14] glutamate transport through the proton-motive force. We have excluded the influence of transport by uncoupling to the same $\Delta\tilde{\mu}_{H^+}$ with a protonophore and the influence of matrix reactions by using submitochondrial particles.

As far as the influence of phloretin on the respiratory rate of isolated rat liver mitochondria is concerned, our results confirm the results reported by Kimura and co-workers [5], although they were unable to demonstrate stimulation of ascorbate oxidation. However, they did not observe any effect of phloretin on the fluorescence of 8-anilino-1-naphthalenesulphonate, which they interpreted as an indication that phloretin does not change the membrane potential. 8-Anilino-1-naphthalenesulphonate, however, is primarily an indicator of surface potentials, as these authors have shown [15]; it may, however, respond secondarily to transmembrane potentials [16]. Therefore, the question remains as to whether transmembrane

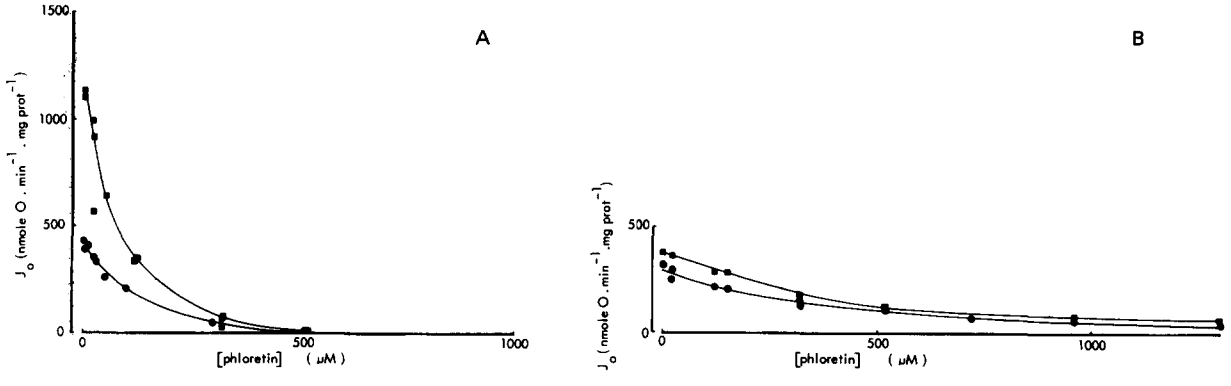

Fig. 2. Effect of different phloretin concentrations on respiration of submitochondrial particles. Submitochondrial particles (0.3 mg protein) were incubated at 298 K in 1.5 ml of a medium containing 250 mM sucrose, 10 mM phosphate buffer (brought to pH 7.3 with Tris base), 5 mM KCl, 5 mM $MgCl_2$ and 0.5 mM EGTA. 0.7 mg/l FCCP was added (■) or omitted (●). Phloretin was added as described in Fig. 1. Further additions were 1 mM NADH (A) or 10 mM Tris-succinate plus 0.7 mg/l rotenone (B).

and surface potentials are still correlated in the presence of phloretin. We measured the transmembrane potential directly and found a simultaneous reduction in transmembrane potential and stimulation of succinate oxidation in State 4. This establishes that stimulation of State 4 oxidation is indeed a consequence of uncoupling.

Since they could not explain the mechanism of the phloretin effect, Kimura and co-workers took refuge in a description in terms of (phenomenological) linear irreversible thermodynamics developed by Caplan and Essig [17]. They postulated different effects of phloretin on the phenomenological coefficients L_{OH} and L_O which should vary with 'Site' and respiratory state. Unfortunately, this description does not provide any information about the mechanism involved and could therefore be better replaced by a description in terms of mosaic non-equilibrium thermodynamics [18,19], which does give such information, making use of the chemiosmotic theory [20] and mechanistic parameters like proton pump stoicheiometries. In the latter description, the equation used by Kimura and co-workers [5]:

$$J_O = L_{OH}\Delta\tilde{\mu}_{H^+} + L_O A_O^{ex} \quad (3)$$

is replaced by

$$J_O = L_O\left(n_H^O \Delta\tilde{\mu}_{H^+} + A_O^{ex}\right) \quad (4)$$

where A_O^{ex} is the free energy difference of the redox reaction (succinate or glutamate oxidation) in the outer medium, n_H^O the number of protons pumped from the matrix to the other medium during the passage of two electrons along the respiratory chain, and L_{OH} and L_O proportionality constants. Eqn. 4 shows directly that the explanation of Kimura and co-workers [5], i.e., different influences of phloretin on succinate oxidation in State 3 and State 4, respectively, is in conflict with the chemiosmotic theory [20], where L_{OH} and L_O differ only in the stoicheimetric number n_H^O [18]. Although we would not reject a priori an explanation on this ground, it can easily be circumvented by the fact that the decrease in L_O by phloretin is more pronounced for glutamate oxidation than for succinate oxidation.

The experiments described in this paper do not provide any information about the mechanism by which phloretin exerts its influence. It is very interesting, however, to note that phloretin is a largely hydrophobic molecule with an ionizable group (pK_a about 7.3 [21]). This implies that it could render the surface potential of the membrane more negative (this was also mentioned by Kimura and co-workers [5]), thereby changing the interactions between the components of the respiratory chain. This mechanism was proposed by Mehlhorn and Packer [22] to explain the effects of sodium dodecyl sulphate on respiring mitochondria and submitochondrial particles; these effects are very similar to those of phoretin. Another possible explanation might be that phloretin lowers the diffusional mobility of membrane proteins, thereby decreasing the rate of electron transfer between the dehydrogenases and Complex III but not between cytochrome *c* and oxygen, according to the findings of Schneider and co-workers [23].

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