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A structure–activity study of fatty acid interaction with mitochondrial uncoupling protein

Petr Ježek[a,*], Martin Modriansky[1,b], Keith D. Garlid[b]

[a]*Institute of Physiology, Department of Membrane Transport Biophysics, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 14220 Prague, Czech Republic*

[b]*Oregon Graduate Institute, Department of Chemistry, Biochemistry and Molecular Biology, 20000 NW Walker Road, Portland, OR 97291-1000, USA*

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Abstract Fatty acid (FA) uniport via mitochondrial uncoupling protein (UcP) was detected fluorometrically with PBFI, potassium-binding benzofuran phthalate and SPQ, 6-methoxy-*N*-(3-sulfopropyl)-quinolinium, indicating K^+ and H^+, respectively. The FA structural patterns required for FA flip-flop, UcP-mediated FA uniport, activation of UcP-mediated H^+ transport in proteoliposomes, and inhibition of UcP-mediated Cl^- uniport by FA, were identical. Positive responses were found exclusively with FA which were able to flip-flop in a protonated form across the membrane and no responses were found with 'inactive' FA lacking the flip-flop ability. The findings support the existence of FA cycling mechanism.

Key words: Reconstitution; Uncoupling protein; H^+ transport; Fatty acid uniport; Chloride uniport; Fatty acid flip-flop

1. Introduction

The mitochondrial uncoupling protein (UcP), expressed in mammalian brown adipose tissue (BAT), is the terminal regulated unit of the catabolic cascade of non-shivering thermogenesis [1,2]. UcP allows for H^+ backflow across the mitochondrial inner membrane in the presence of free fatty acids (FA), thus dissipating the protonmotive force and generating heat [1,2]. The consensus was that the free FA participate in the process, but FA were considered to act as a buffering agent without being transported themselves [1]. On the contrary, we have published evidence recently [3–7] supporting the mechanism of fatty acid cycling hypothesized by Skulachev [8]. UcP is regarded as an anion channel which transports FA anions unidirectionally. Consequently, H^+ translocation originates from the return of protonated FA through the lipid bilayer. Such a mechanism would be possible under the following circumstances. (1) UcP must be an anion uniporter; this condition is fulfilled [9,10], but the structural requirement for the monovalent non-carboxylic substrates and their maximum size is yet to be investigated. (2) UcP must transport anionic FA. Such a uniport (apparent as the H^+ counterflux due to the presumed FA cycling) should exhibit the properties very similar to the uniport of other UcP substrates, since the internal binding site mediating translocation, a 'docking site' [10], was found to be common for anions and FA [3–7]. (3) Non-ionic FA in the membrane must undergo a rapid flip-flop that would balance the reported activities of UcP, i.e., account for at least 11–22 μmol H^+/min·mg protein which equals to 726–1452 min^{-1} turnover for a dimeric UcP [3,5,6,11,12]. The actual measurements of FA flip-flop [13–15] already showed that this can be true.

Garlid et al. [6] demonstrated that a pair of similar amphiphiles, undecanesulfonate and laurate, can both undergo a GDP-sensitive uniport mediated by UcP, but only laurate in its protonated form is capable of rapid flip-flop across the liposomal membrane. The flip-flop results in acidification of the liposomal interior after H^+ dissociate from laurate at the inner surface.

Moreover, the GDP-sensitive H^+ flux can be induced only with laurate, but not with undecanesulfonate, in proteoliposomes containing UcP. By applying Occam's razor the laurate-induced UcP-mediated H^+ flux was explained by the FA cycling mechanism [6].

In the accompanying paper [15] we report existence of 'inactive' FA, the FA derivatives that were unable to flip-flop across the lipid bilayer. The inactive FA share the inability to flip-flop with alkylsulfonates even though the carboxy group is preserved. In this work we tested how the inactive FA interact with reconstituted UcP. We have found a strong correlation between the inability of FA to flip-flop, its inability to be transported by UcP and to induce UcP-mediated H^+ translocation, and with the lack of FA inhibition of Cl^- transport via UcP. Such correlation supports the FA cycling mechanism.

2. Materials and methods

The fluorescent probes PBFI and SPQ were purchased from Molecular Probes (USA). Various derivatives of FA were purchased from Sigma (USA) and Fluka (Germany) or Lancaster (UK). The materials for reconstitution were from the same sources as previously described [3,6,9,10,16]. BAT mitochondria were isolated from Syrian hamsters in 250 mM sucrose, 10 mM TEA-TES, 1 mM K-EGTA, pH 6.7, containing 2 mg BSA/ml. Reconstitution of UcP was performed and H^+ and K^+ fluxes were measured fluorometrically using a SLM 8000C fluorometer as described elsewhere [3,6,9,10]. The transport rates were calculated in mM H^+/s. The amount of reconstituted UcP was estimated by Amido black method [17] and the volume of vesicles was determined from probe distribution [3,6]. FA were added as μl aliquots of ethanol solutions.

*Corresponding author. Fax: (420) 2-4719517.
E-mail: jezek@sun1.biomed.cas.cz

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Abbreviations: BAT, brown adipose tissue; FA, free fatty acids (non-esterified); Octyl-POE, octylpentaoxyethylene; PBFI, potassium-binding benzofuran phthalate; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; TEA, tetraethylammonium-; TES, *N*-tris [hydroxymethyl]-methyl-2-aminoethane-sulfonic acid; TNP, trinitrophenyl-; UcP, uncoupling protein

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2.1. 'SPQ quenching' method for indication of H^+ fluxes

SPQ is quenched by TES^- anion but not by TES zwitterion [18] which allows for monitoring of H^+ fluxes after calibration by 1 N KOH additions in the presence of nigericin [6,18]. Vesicles (25 μl/assay) contained 84.4 mM TEA_2-SO_4, 29 mM TES (TEA-salt), pH 7.2, 0.6 mM TEA-EGTA and 2 mM SPQ. External medium contained 84.4 mM K_2SO_4, 29 mM TES (TEA), pH 7.2, and 0.6 mM TEA-EGTA. UcP-mediated H^+ efflux in the presence of FA was initiated by 0.1 μM valinomycin.

2.2. PBFI detection of FA uniport

A K^+ gradient is established across the liposomal membrane and used for driving the UcP-mediated transport of FA anions. The K^+ uptake can be used as an indirect measurement of UcP-mediated transport by assuming it is equivalent to the net charge flux facilitated by UcP. The process is initiated by addition of 0.1 μM valinomycin, while internal $[K^+]$ is detected by following PBFI fluorescence [19]. The system is calibrated by adding aliquots of 2 M KCl in the presence of 0.5 μM nigericin and 5 μM tributyltin. 25 μl of vesicles were used, interior of which contained 75 mM TEA_2-SO_4, 75 mM TEA-TES, pH 7.2, 0.05 mM K_2SO_4 and 300 μM PBFI. The external medium (1.975 ml) contained 75 mM K_2SO_4, 75 mM TEA-TES, pH 7.2.

2.3. SPQ detection of Cl^- uptake

Vesicles (25 μl in cuvette) contained 2 mM SPQ and 79.4 mM TEA_2-SO_4, 0.6 mM TEA-EGTA and Tris-P_i, pH 7.2 (24.7 mM Tris, 12.8 mM P_i). External medium for higher Cl^- gradient contained 119.25 mM KCl, 0.6 mM TEA-EGTA and Tris-P_i (as above), pH 7.2. Alternative external medium had only 74.8 mM $[Cl^-]$, but $[K^+]$ was 201.2 mM in 126.4 mM glucuronate and 32.5 mM SO_4^{2-} salts and 0.6 mM TEA-EGTA and Tris-P_i (as above) pH 7.2, were also present. Cl^- uptake was initiated by 0.1 μM valinomycin. Calibration of SPQ quenching by 2 M KCl was done in the presence of 0.5 μM nigericin and 5 μM tributyltin [9]. Experimentally measured fluorescence trace were transferred into 'Cl-traces' by iterations for L according the equation:

$$[Cl^-]_{(t)} = m^{-1} \cdot (F_0^{exp} - F_{(t)})/(F_{(t)} - L) \quad (1)$$

where L is light scattering (i.e. background) of a given sample; F_0^{exp} is intercept of calibration (F_0, maximum fluorescence intensity at zero $[Cl^-]$) corrected for light scattering; and m is experimental Stern-Volmer constant of calibration (usually 0.060 mM^{-1}).

3. Results

Net H^+ flux in vesicles can be monitored by following the quenching of SPQ fluorescence by TES^- anion [18]. Fig. 1 (trace +UCP) illustrates the internal acidification of proteoliposomes containing UcP after addition of 50 μM heptylbenzoic acid. The non-ionic form of FA can flip to the inner lipid leaflet and acidify the vesicle interior by releasing the carboxy proton [15]. The same phenomenon is also observed in liposomes lacking UcP (Fig. 1, trace −UCP). Addition of valinomycin induces internal alkalization of vesicles containing UcP, thus indicating the protein dependent H^+ efflux, because such an alkalization is not observed in liposomes lacking UcP. The FA-induced H^+ leak in liposomes is negligible [15].

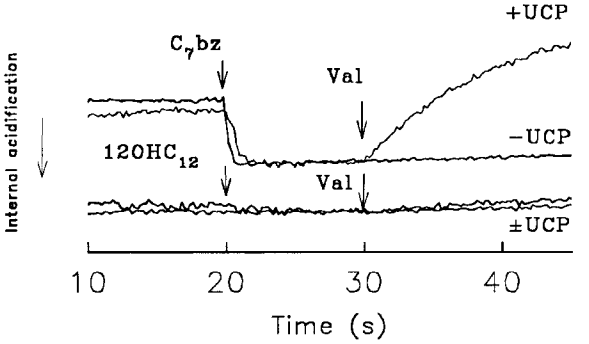

Fig. 1. Fatty acid cycling requires presence of the uncoupling protein and the active fatty acid. Net H^+ fluxes in proteoliposomes containing UcP (+UCP) and in liposomes (−UCP) were monitored as quenching of SPQ fluorescence by TES^- anion [19]. Either, 100 nmol of the 'active' FA, heptylbenzoic acid (C_7bz) or the 'inactive' FA, 12-hydroxylauric ($12OHC_{12}$) were added (50 μM final concentrations) as indicated by the arrows. 0.1 μM valinomycin (Val) was added at 30 s.

The H^+ flux in UcP proteoliposomes is sensitive to GDP[2,3] (Fig. 2), confirming the participation of UcP [3,6]. Our interpretation attributes the observed H^+ efflux to the return of the protonated FA while the UcP mediates influx of the anionic FA [6]. The ability to acidify vesicle interior and subsequent valinomycin-induced GDP-sensitive H^+ efflux was observed with various FA derivatives (Fig. 2 and Table 1). Magnitudes of flip-flop rates correlated with the rates of H^+ efflux.[2] Nevertheless, as reported in the accompanying paper [15] we found several FA derivatives, which did not acidify vesicle interior, i.e. were unable to flip-flop. These 'inactive' FA did not show either the UcP related H^+ efflux (Figs. 1 and 2 and Table 1). Typically, they were bipolar compounds such as 12-hydroxylauric acid, 12-dodecanedioic acid or compounds with a benzene ring at the tail, such as phenylvaleric acid. The inactive FA had no influence on the transport induced by the 'active' FA. For example, H^+ efflux induced by lauric acid in UcP proteoliposomes was neither inhibited, nor increased by addition of 12-hydroxylauric acid before lauric (not shown). H^+ transport, induced by all active FA tested, was inhibited by 150 μM undecanesulfonate, as reported previously for laurate [6].

Net charge movement in vesicles can be measured with fluorescent probe PBFI [6,19]. With various active FA added to the UcP proteoliposomes, a GDP-sensitive K^+ uptake was observed[3]. Such an uptake was not observed with the inactive FA (Fig. 3 and Table 1). Rates of FA uniport correlated[2] with the rates of the H^+ efflux induced by a given FA (Table 1).

Net Cl^- uptake can be measured with SPQ in vesicles containing no TES but P_i, while Cl^- acts as an SPQ quencher [18]. The Cl^- transport in UcP proteoliposomes is competitively inhibited by lauric acid [3] and we observed a strong inhibition caused by various FA (Fig. 4 and Table 1). With no exception, inhibitory ability was pronounced only with active FA, some of which inhibited the Cl^- uniport via UcP nearly completely (Table 1). The inactive FA were unable to inhibit the Cl^- uniport through the reconstituted UcP (Fig. 4 and Table 1). Inhibitory strength of active FA correlated[2] with their relative activity in other tests (Table 1).

4. Discussion

The coherence of structural patterns of FA required for *trans*-membrane flip-flop and interaction with the UcP (Table

[2] Our results have shown that the resulted effects of FA are invariant of the data treatment when either net rates or GDP-sensitive rates were taken into account.

[3] Resulted UcP-proteoliposomes [6,9,10] have equal orientation distribution with respect to the exposure of UcP GDP-binding site outward or inward. Hence, GDP added externally causes maximum 50% inhibition, added in both sides, inhibits completely [6,9,10]. Therefore, every test was performed in pairs with and without external GDP (calibrated separately) and their differences were taken as the net GDP-sensitive fluxes.

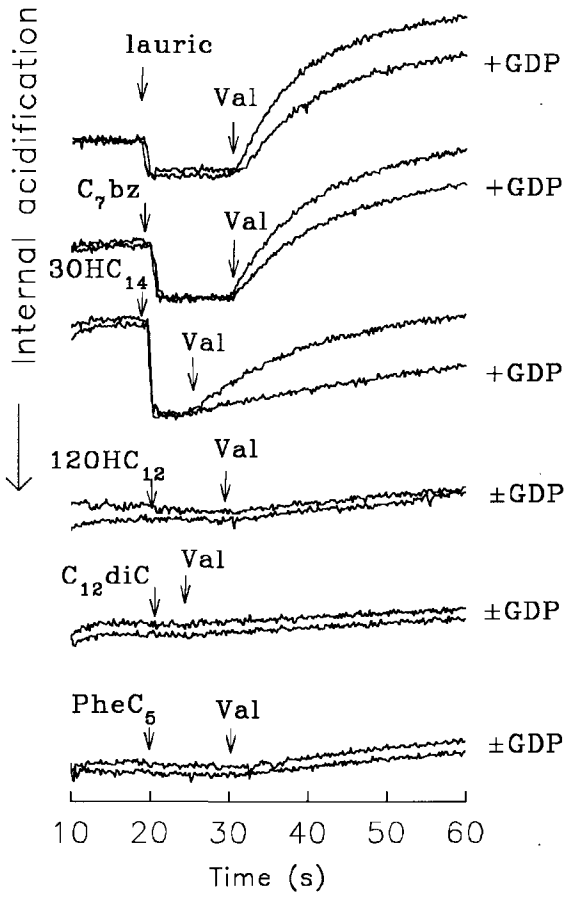

Fig. 2. GDP inhibition of fatty acid cycling. Net H^+ fluxes in proteoliposomes containing UcP were monitored as quenching of SPQ fluorescence by TES^- anion [19]. Each panel shows a pair of traces expressed as δH^+ vs. time, measured in the absence (no label) or presence of 0.5 mM GDP (traces +GDP), when 50 μM fatty acid was added at 20 s and 0.1 μM valinomycin (Val) at 30 s (or 25 s) to initiate UcP mediated transport. Top half contains traces obtained *in the presence of active FA*, lauric, heptylbenzoic (C_7bz) and 3-hydroxymyristic (3-OHC_{14}) acids; bottom-half contains traces obtained in the presence of *inactive FA*, 12-hydroxylauric ("12OHC_{12}"); dodecanedioic (C_{12}diC) and phenylvaleric ($PheC_5$) acids.

1) is consistent with the FA cycling mechanism [6,8]. Cycling of the active FA is given by the ability of UcP to translocate its anionic form (Fig. 3) and by the ability of active FA to flip-flop in a protonated form (Figs. 1 and 2) which leads to H^+ translocation. This FA protonophore model for the mechanism of UcP-mediated H^+ transport [6] requires that UcP translocates the anionic FA carboxylate headgroup from one side of the membrane to the other. The FA anion transport is driven by respiratory H^+ pumps and can be facilitated by an internal energy well in the transport pathway of UcP located near the center of the membrane [6,10]. H^+ are then delivered by non-ionic flip-flop of the neutral FA back to the other side. Thus, the role of UcP is to enable FA to act as cycling protonophores in BAT mitochondria [3,6]. This mechanism has certain advantages, not least of which is providing an explanation for the long-standing observation that UcP transports a variety of monovalent anions besides mediation of H^+ transport [3,6].

The existence of inactive FA that are unable to flip-flop and unable to activate UcP mediated H^+ transport supports the existence of FA cycling mechanism. 'Inhibited' flip-flop with the inactive FA could clearly prevent the whole FA cycling. Moreover, we have shown that the pathway of FA uniport and the pathway of protonated FA both must be present. The uniport pathway is not used by the inactive FA (they are not transported by UcP; Fig. 3) since they do not show a charge compensation by K^+ in UcP proteoliposomes. Also, no UcP-related H^+ efflux was induced with the inactive FA. Consequently, we must exclude a possibility that such an H^+ efflux can be allosterically activated, either from a 'docking site' [5,10] or by a mechanism of local buffering suggested by Klingenberg [1,12]. If such mechanism existed, the inactive FA should provide local buffering of the same strength as the active FA.

The inabilities of the inactive FA to flip-flop and to reach the internal anion binding site of UcP (energy well) are closely related. The latter can be deduced simply from the lack of their uniport via UcP, but it is reflected more directly by the lack of inhibition of transport of Cl^- and other anions by UcP. On the contrary, the active FA competitively inhibit uniport of Cl^- [3,6] and alkylsulfonates [6] via UcP. To achieve this inhibition the active FA have to interact with the internal binding site [6]. The inactive FA have mostly bipolar character or have a terminal phenyl. We have demonstrated that they do partition into the bilayer [15] and hypothesized that their inability to flip-flop is given by a non-standard conformation, for example by a U-shape, when present within a lipid bilayer. This might also explain why they do not interact with UcP, since the unusual conformation would prevent the inactive FA from reaching the internal translocation site of UcP, which might be formed by a cluster of positively charged or hydrophobic amino acid residues.

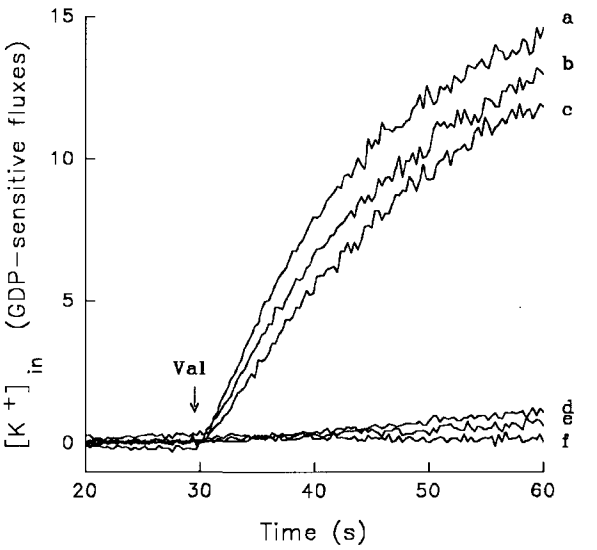

Fig. 3. GDP-sensitive fatty acid uniport proceeds with the active but not with the inactive fatty acids. Fatty acid anion uptake (or concomitant H^+ efflux via protonated FA) was detected using PBFI as K^+ uptake into proteoliposomes containing PBFI. Transport was initiated by adding 0.1 μM valinomycin. The 'GDP-sensitive' 'K-traces' were constructed from PBFI-fluorescence recorded for each sample in the absence of GDP and in the presence of 0.5 mM GDP[3]. The differential K-traces are shown for *active FA*, lauric (trace a), 3-hydroxymyristic (b) and 12-hydroxystearic (c) acids, and for the *inactive FA*, 12-hydroxylauric (d), dodecanedioic (e) and phenylvaleric (f) acids.

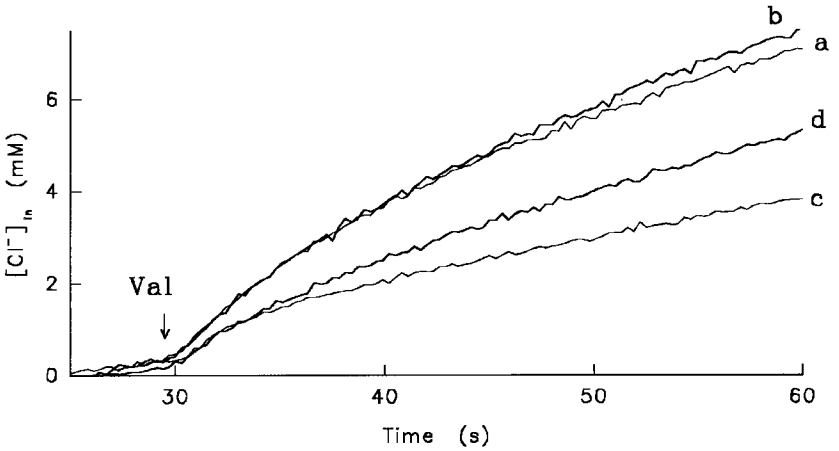

Fig. 4. Inhibition of Cl^- uptake into proteoliposomes containing UcP by the active fatty acids and its absence with the inactive fatty acids. Cl^- transport was detected as quenching of SPQ fluorescence by Cl^- in TES-free media containing Tris-P_i buffer. Traces show Cl^- transport in the absence of added FA (trace a, control) and in the presence of 200 μM 12-hydroxylauric (b), lauric (c), and heptylbenzoic (d) acids. % Inhibition of the initial rate was estimated as 0% (b), 65% (c) and 57% (d). When GDP-sensitive parts were compared (traces with GDP are not shown), the resulted inhibition reached 0%, 88% and 97%, respectively.

Physiologically, UcP attracts to this energy well the negatively charged carboxyls [6] of the active, naturally abundant, FA [20] which possess the unipolar character. By this interaction UcP discharges all 'polarities' and the non-polar hydro-

Table 1
Effects of active and inactive fatty acids in proteoliposomes containing UcP (GDP-sensitive fluxes) and in liposomes

	Liposomes:	Proteoliposomes:		
	flip-flop acidification	UCP-mediated H^+ transport	UCP-mediated charge transport	Inhibition of Cl^- transport
1. Active fatty acids	**yes**	**yes**	**yes**	**yes**
Lauric	100%	100%	100%	78%, 90%[a]
2-Hydroxylauric	75%	58%	135%	66%
12-Bromo-lauric	37%	72%	116%	40%
12-TNT-lauric[b]	9–19%		87%	58%
Myristic	93%	140%	112%	81%
2-Hydroxymyristic	370%	149%	136%	64%
3-Hydroxymyristic	124%	86%	83%	50%, 99%[a]
Palmitic	83%	134%	112%	73%
2-Hydroxypalmitic	68%	149%	150%	75%
Stearic	15%	30%	33%	25%
2-Hydroxystearic	23%	154%	70%	52%
12-Hydroxystearic	75%	67%	81%	17%, 61%[a]
Heptylbenzoic	107%	68%	90%	60%, 97%[a]
Dodecyloxybenzoic	16%	21%	30%	n.d.
16-Hydroxypalmitic	75%	27%	49%	7%
Hexadecanedioic	37%	17%	27%	9%
2A. Inactive fatty acids	**no**	**no**	**no**	**no**
12-Hydroxylauric	0.01%	0%	4%	0%, 1%[a]
12-Aminolauric	0.01%	4%	3%	0%
Dodecanedioic	0.3%	3%	2%	0%, 1%[a]
Tetradecanedioic	0.003%	7%	16%	0%
Phenylhexanoic	0.03%	2%	6%	7%
Phenylvaleric	0.003%	3%	0.5%	0%, 0.4%[a]
Aleuritic	0.16%	1.7%	1.2%	0%
Biphenyl-2-carboxylic	0.03%	3.4%	4.7%	8%
3,3-Diphenylpropionic	0.003%	4.6%	5.3%	0%
2-Naphtoic	0.03%	5.3%	6.4%	0%
Ethanol	0%	5%	6%	0–5%
2B. Inactive fatty acids	**yes**	**no**	**no**	**no**
None found				
3. Transported anions	**no**	**no**	**yes**	**yes**
Undecanesulfonate	0%	0%	200%	98%[a]
4. Model violation	**no**	**yes**	**yes**	**yes/no**
none found				

For comparison of effects of 50 μM FA we set magnitudes of flip-flop rate or GDP-sensitive transport rates as 100%. The % inhibition of Cl^- uniport by FA is listed in absolute values.
[a]200 μM FA instead of 50 μM.
[b]10 μM.

phobic tail may emerge into the hydrophobic bilayer core as does the neutral active FA during flip-flop in the lipid bilayer. Structural requirement for FA to be UcP substrate (Table 1) is similar as that found for the non-carboxylic substrates of UcP [10]. (i) The monovalent charge is strictly required. (ii) The unipolarity is required — an additional polar group might be present only in the closer position to the charge of carboxyl or must be 'shielded' by the additional hydrophobic chain or by a non-polar group. (iii) Even bulky groups are allowed along the aliphatic chain or at its end, provided that they do not bear polar groups or charges. Thus, 'Good' buffer type anions are not UcP substrates [10], whereas groups like nitroxy- of DOXYL [16], nitro-, bromo- are tolerated, as well as the secondary amino group (-NH-) [5]. Benzene ring is tolerated either in the closest position to the carboxyl or when is 'shielded' by nitro- group (TNP-lauric, or compounds described in [5,7]). Contrary to Klingenberg [12] we found that both phenylvaleric and phenylhexanoic acid are inactive (Figs. 2–4 and Table 1). A 'mirror' derivative, heptylbenzoic acid, was one of the most active.

The previous evidence for FA cycling mechanism includes the comparison of behavior of laurate and its close analogue, undecanesulfonate [3,6]. The single difference that may explain the failure of undecanesulfonate to deliver H^+ electroneutrally by flip-flop is it being a very strong acid. We may regard the inactive FA as similar amphiphiles which lack the flip-flop ability as undecanesulfonate does. However, the important difference is that the latter is transported by UcP while the inactive FA are not.

Our indirect evidence for the FA cycling is currently the best available. It is not possible to measure FA anion transport directly, because flip-flop equilibration of the acid is a fast process and internal accumulation of FA anions never occurs. It is, however, possible to refute the model, which was one of the rationale for the presented series of experiments. The FA protonophore model would become invalid, if an FA analogue is found that catalyzes UcP-mediated H^+ transport without being able to acidify the liposome interior by flip-flop (case 4, Table 1). The important outcome of this study is that no such violation has been found to this date. A second motivation for the study was to develop some understanding of the structure-activity relationships that exist among the vast family of FA and their derivatives and analogues. This has led to some interesting results. The dominant indicator of 'active FA', i.e. those able to induce UcP-mediated H^+ flux, is the ability to deliver protons by flip-flop to the intraliposomal medium. Flip-flop was always associated with the ability to induce UcP-mediated H^+ flux. It would not violate the model to have found FA capable of flip-flop, but unable to induce UcP-mediated H^+ transport. We have found none.

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