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Alkylsulfonates as Probes of Uncoupling Protein Transport Mechanism

ION PAIR TRANSPORT DEMONSTRATES THAT DIRECT H^+ TRANSLOCATION BY UCP1 IS NOT NECESSARY FOR UNCOUPLING*

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The mechanism of fatty acid-dependent uncoupling by mitochondrial uncoupling proteins (UCP) is still in debate. We have hypothesized that the anionic fatty acid head group is translocated by UCP, and the proton is transported electroneutrally in the bilayer by flip-flop of the protonated fatty acid. Alkylsulfonates are useful as probes of the UCP transport mechanism. They are analogues of fatty acids, and they are transported by UCP1, UCP2, and UCP3. We show that undecanesulfonate and laurate are mutually competitive inhibitors, supporting the hypothesis that fatty acid anion is transported by UCP1. Alkylsulfonates cannot be protonated because of their low pK_a, consequently, they cannot catalyze electroneutral proton transport in the bilayer and cannot support uncoupling by UCP. We report for the first time that propranolol forms permeant ion pairs with the alkylsulfonates, thereby removing this restriction. Because a proton is transported with the neutral ion pair, the sulfonate is able to deliver protons across the bilayer, behaving as if it were a fatty acid. When ion pair transport is combined with UCP1, we now observe electrophoretic proton transport and uncoupling of brown adipose tissue mitochondria. These experiments confirm that the proton transport of UCP-mediated uncoupling takes place in the lipid bilayer and not via UCP itself. Thus, UCP1, like other members of its gene family, translocates anions and does not translocate protons.

Five distinct genes for mitochondrial uncoupling proteins (UCP1–5)[1] have been recognized in the human genome, and three distinct genes have been identified in the *Arabidopsis* genome (1). Those UCPs that have been characterized with respect to transport function share a fundamental phenotype, they catalyze FA-induced proton uniport (2–6). To explain the mechanism of FA-induced uncoupling, two major hypotheses have been advanced that differ primarily in the site through which protons are translocated. Klingenberg's group proposes a FA Buffer Model, in which the proton translocation pathway resides in the UCP structure (7–9). FAs are somehow bound to the protein and focus protons into the pathway by protonation-deprotonation. We propose a FA protonophore model in which the UCPs translocate the anionic FA head group from the matrix to the outer surface of the inner membrane. The FA carboxylate then picks up a proton and rapidly flip-flops back, to release the proton into the matrix (2, 4, 10–14). The UCPs thus catalyze a regulated protonophoretic cycle in which FA, and not UCP protein, serves as the carrier for protons.

The FA protonophore model integrates uncoupling with the known anion transport function of UCPs (2, 15–19), thereby conforming to the transport function of the other members of the gene family of mitochondrial anion carriers (20). Indeed, the hypothesis developed out of the recognition that a particular class of anions, the long-chain alkylsulfonates, are ideal probes of UCP transport mechanism. We have studied sulfonates ranging in chain length from 1 to 16 (2, 18), but we have focused on C11-sulfonate, which is transported by UCP1 (2, 18), UCP2 and UCP3 (4), and plant UCP (3). C11-sulfonate is identical to laurate except for the head group, and it is transported with K_m and V_{max} values close to those obtained for laurate-dependent H^+ transport (2). Nevertheless, C11-sulfonate cannot catalyze proton transport, because its very low pK_a prevents its flip-flop with protons across the lipid bilayer (2). Therefore, C11-sulfonate only participates in the half-cycle of uncoupling that involves UCP. Our basic argument on behalf of the model is that the UCPs contain a transport pathway for long-chain alkylsulfonates, and there is no known mechanism to prevent FA anion from using that pathway.

This paper extends our studies of alkylsulfonates as probes of UCP transport mechanism. We describe two new protocols to drive UCP-mediated translocation of the C11-sulfonate head group across the membrane. We show that laurate and C11-sulfonate are mutually competitive substrates of UCP1, indicating that they share a common anion transport mechanism. We show that C11-sulfonate transport and laurate-dependent H^+ transport are inhibited by GDP with the same K_i. We also report for the first time that C11-sulfonate can be electroneutrally transported with a proton across the membrane as an ion pair with propranolol, an amphiphilic base. Ion-pair transport

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[1] The abbreviations used are: BAT, brown adipose tissue; BSA, bovine serum albumin; C11-sulfonate, undecanesulfonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FA, fatty acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PBFI, potassium-binding benzofurane isophtalate; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; TEA^+, tetraethylammonium cation; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; UCP, uncoupling protein; UCP1, brown adipose tissue-specific UCP; MES, 4-morpholinoethanesulfonic acid.

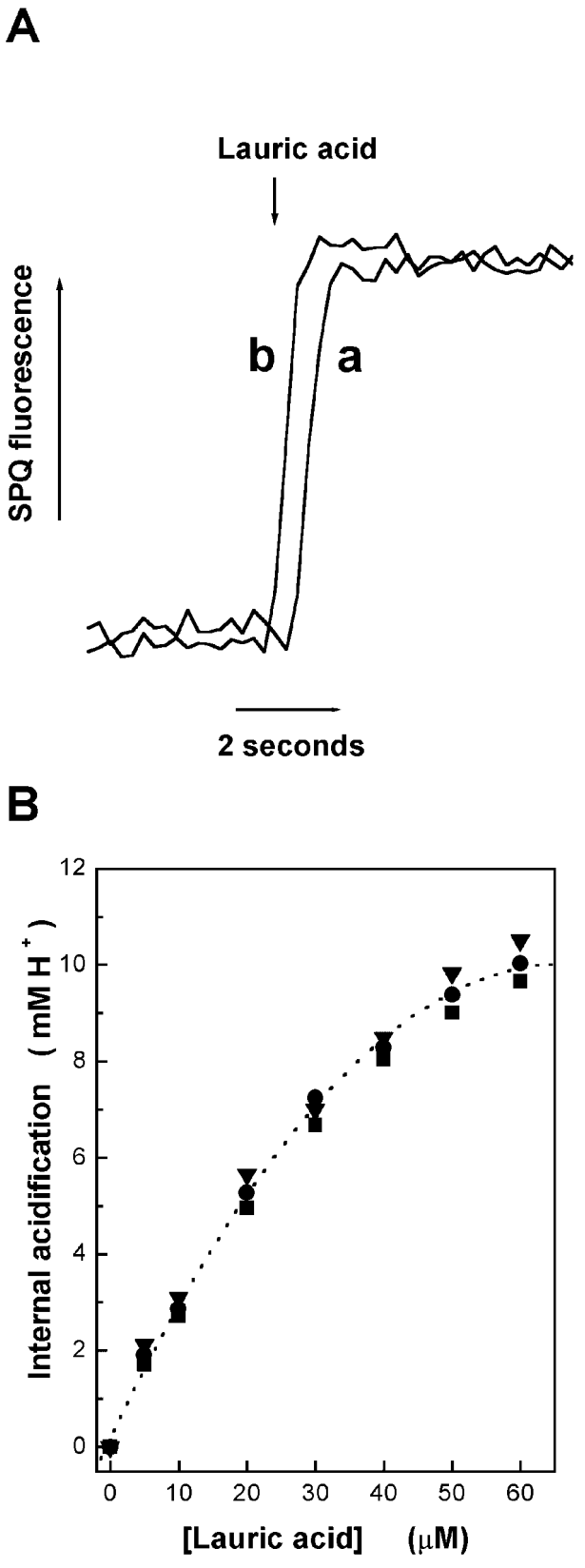

FIG. 1. **C11-sulfonate does not affect flip-flop acidification by lauric acid in liposomes.** *Panel A,* representative fluorescent traces of flip-flop acidification induced by 50 μM lauric acid in the absence (*trace a*) and presence (*trace b*) of 100 μM C11-sulfonate. *Panel B*, concentration dependence of laurate-induced extent of flip-flop acidification, ("internal acidification") in the absence (■) or presence of 50 μM (●) and 100 μM (▼) C11-sulfonate. Protein-free liposomes were prepared and H^+ influx was measured using SPQ quenching method in the presence of K^+ gradient, as described under "Experimental Procedures."

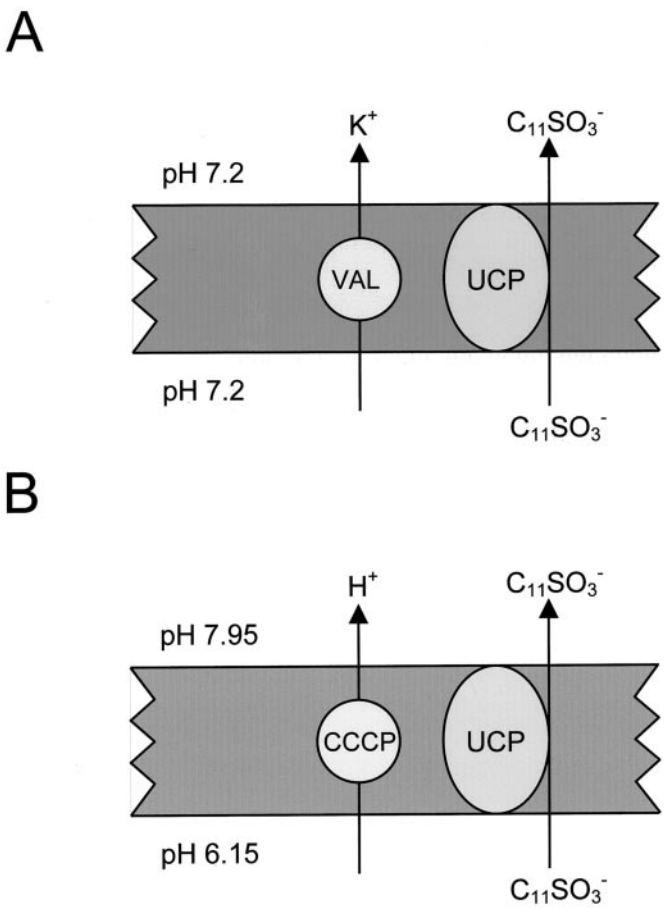

FIG. 2. **C11-sulfonate uniport through UCP may be driven either by K^+ gradient (*panel A*) or by pH gradient (*panel B*).** Upon addition of C11-sulfonate, the head group will equilibrate across the membrane, via UCP, in a small capacitative discharge. No further C11-sulfonate transport will occur until a compensating ion flux is added. This can be provided either through valinomycin in the presence of a K^+ gradient, as shown in *panel A*, or through CCCP in the presence of a pH gradient, as shown in *panel B*. When valinomycin is used, net influx of the C11-sulfonate head group must equal net influx of K^+, which may be followed by PBFI fluorescence. When CCCP is used, net influx of the C11-sulfonate head group must equal net influx of H^+, which may be followed by SPQ quenching.

enables C11-sulfonate to uncouple BAT mitochondria and also to catalyze GDP-dependent, electrophoretic proton transport in proteoliposomes. These results provide new, independent evidence that anion translocation by UCP1 is necessary for uncoupling, whereas proton transport occurs spontaneously in the bilayer.[2]

EXPERIMENTAL PROCEDURES

Mitochondrial Preparations—BAT mitochondria were isolated from Syrian hamsters as described previously (21). For studies on intact mitochondria, isolation medium contained 250 mM sucrose, 10 mM MOPS, 1 mM EDTA, 1 mM dithiothreitol, and 2 mM ATP, adjusted to pH 7.4 at 25 °C with Tris base. During the final centrifugation, the isolation medium was supplemented with BSA (2 mg/ml), and ATP and dithiothreitol were omitted. Mitochondria were suspended at 50 mg of protein/ml in isolation medium lacking BSA, ATP, and dithiothreitol. BSA treatment was omitted when mitochondria were prepared to undergo endogenous FA removal using the carnitine cycle (22). For reconstitutions, isolation medium contained 2 mg/ml BSA. The mitochondria were treated with additional BSA as follows: the mitochondrial pellet was suspended in 0.75 ml of isolation medium containing 100 mg/ml BSA and incubated for 5 min at room temperature. The volume was adjusted to 30 ml with isolation medium and mitochondria were col-

[2] A preliminary report of these results was presented in abstract form (37).

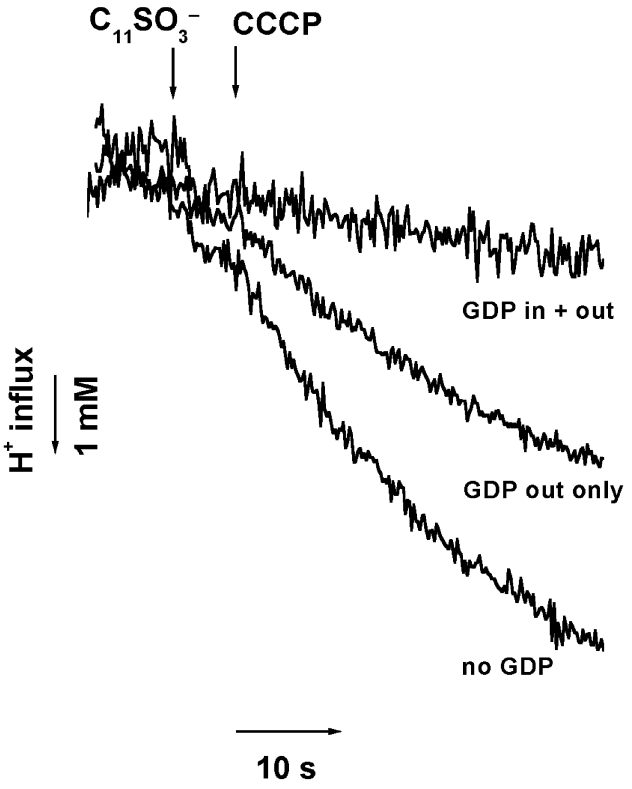

FIG. 3. **GDP-dependent C11-sulfonate uniport through UCP1 driven by pH gradient in the presence of CCCP.** Shown are traces of charge transport, measured as total acid influx, as monitored by SPQ fluorescence. Addition of 100 nM CCCP (*CCCP*) caused a rapid internal acidification (*no GDP*), reflecting inward net transport of the C11-sulfonate head group, as diagrammed in Fig. 2*B*. C11-sulfonate uniport was partially inhibited by 1 mM external GDP (*GDP out only*) and completely inhibited when 1 mM GDP was present on both sides of the membrane (*GDPin+out*). Proteoliposomes were prepared and pH gradient media were used, as described under "Experimental Procedures." This experiment is representative of three similar experiments.

lected by centrifugation at 20,800 × *g* for 10 min. This step was repeated twice. The final pellet was resuspended in isolation medium and mitochondria were stored in aliquots of 7.5 mg of protein at −20 °C.

Purification and Reconstitution of UCP1—UCP1 was purified and reconstituted into proteoliposomes using previously described procedures (2, 21). Briefly, frozen mitochondria were first washed with additional BSA (5 mg/ml) and then extracted with octylpentaoxyethylene in the presence of phospholipids. UCP1 was purified using the hydroxyapatite, Bio-Gel (Bio-Rad). The composition of phospholipids and internal medium were adjusted, and the protein/lipid mixture was incubated with Bio-Beads SM-2 (Bio-Rad) to remove detergent and form vesicles. External probe was removed by passage through Sephadex G-50–300 spin columns, which were pre-equilibrated with internal medium. Protein-free liposomes were prepared using the same protocol.

Fluorescence Monitoring of Ion Fluxes—Ion fluxes in proteoliposomes were measured using ion-specific fluorescent probes and a SLM Aminco 8000C spectrofluorometer. H^+ ion fluxes were measured directly as changes in intraliposomal acid concentration, obtained from changes in SPQ fluorescence due to quenching by TES^- anion (21, 23). K^+ fluxes were obtained from changes in fluorescence of PBFI due to complexation with K^+ (21, 24).

Media compositions were designed to establish electrochemical gradients of K^+ and H^+ to drive transport of anions or H^+ in the desired direction when valinomycin or CCCP were added. When fatty acid or alkylsulfonate transport driven by K^+ diffusion potential was studied, the internal medium contained TEA^+ salts and external medium contained K^+ salts of TES buffer (30 mM, pH 7.2), SO_4 (80 mM), and EGTA (0.6 mM). Transport was initiated by the addition of 25–100 nM valinomycin. When alkylsulfonate transport driven by pH gradient was studied, internal medium contained TEA^+ salt of TES buffer (22.5 mM, pH 7.95), and the external medium contained TEA^+ salt of MES buffer (30 mM, pH 6.15). Both media contained TEA^+ salts of SO_4 (80 mM) and EGTA (0.6 mM). Transport was initiated by the addition of 100 nM CCCP.

Each preparation was individually calibrated for fluorescence probe response, and the internal volume of vesicles was estimated from the volume of distribution of the fluorescent probe (21). Protein content of liposomes was estimated by the Amido Black procedure (25). Background leaks were estimated from the flux in the presence of saturating GDP (1 mM) in the intra- and extraliposomal media and used to correct the observed fluxes. Kinetic parameters referred to under "Results" were derived from the Hill equation fits to the corrected fluxes.

Respiration and Potential Monitoring of BAT Mitochondria—Assay medium contained 50 mM KCl, 4 mM KP_i, 2 mM $MgCl_2$, 1 mM EDTA, 10 μM BSA, 0.2 mM ATP, 0.25 μM cyclosporin A, 5 mM pyruvate, 5 mM malate, and 20 mM Tris-MOPS, pH 7.2. α-Glycerolphosphate could not be used as a substrate because low concentrations of C11-sulfonate inhibited the dehydrogenase (not shown). Prior to addition of substrates, endogenous FAs were removed by the carnitine cycle, which was initiated by the addition of 25 μM coenzyme A, 0.5 mM L-carnitine, and 0.2 mM ATP.

Oxygen consumption of 1 mg ml^{-1} BAT mitochondria was estimated using a Clark polarographic oxygen probe (Yellow Springs) in a thermostated chamber at 25 °C. $\Delta\Psi$ was estimated using 2.5 μM Safranin O and 0.5 mg ml^{-1} BAT mitochondria at excitation/emission wavelengths of 495/576 nm, 8-nm slit width.

Chemicals and Reagents—SPQ and PBFI were purchased from Molecular Probes, Inc. Alkylsulfonates were purchased from Research Plus, Inc. Sulfuric acid was purchased from Fisher. All other chemicals were obtained from Sigma or from sources listed previously (2).

RESULTS

Do Long-chain Alkylsulfonates Remove Fatty Acid from the Membrane?—We routinely examine transport in liposomes to distinguish nonspecific effects of compounds used to study UCP. Fig. 1 contains data that address the concern that C11-sulfonate may cause "competitive removal" of FA from the liposomal membrane (7, 8). We examined the rate and extent of the flip-flop acidification that always occurs when lauric acid is added to liposomes (2, 4). FA partitions into the membrane, and the protonated head groups undergo flip-flop until equilibrium is reached (26–28). Both the rate and extent of total acid delivery, recorded by the SPQ probe, will depend on the amount of FA in the membrane. The traces in Fig. 1*A* show that laurate causes release of quenching, indicating interior acidification. The rate of flip-flop equilibration is very rapid, being about 80% complete within 10 ms (29). As can be seen in Fig. 1*A*, the laurate flip-flop rate is not affected by the presence of 100 μM C11-sulfonate. The data in Fig. 1*B* show that the *extent* of proton delivery due to lauric acid flip-flop is also unaffected by C11-sulfonate. Thus, long-chain alkylsulfonates have no significant effect on either the rate or extent of proton delivery. From these results, we may safely conclude that alkylsulfonates neither remove FA from the membrane (7, 8) nor do they affect FA partitioning. Indeed, lack of interference is the expected result, since 50 μM lauric acid in a suspension containing 0.5 mg of lipid ml^{-1} represents a mole fraction in the membrane of only 0.054, assuming a partition coefficient of 5.10^3 (30).

The Head Group of C11-sulfonate Is Transported Across the Membrane by UCP1—Because long-chain alkylsulfonates have a high partition coefficient in the membrane, the head group moves across the membrane, and the hydrophobic alkyl tail remains in the hydrophobic barrier, as it does with FA (2). The conclusion that the head groups of long-chain alkylsulfonates are transported by UCP1 has been challenged on the basis that "C11-sulfonate . . . did not pass through UCP1 but was driven through the membrane . . . in a ternary complex formed with valinomycin and K^+" (7, 8). We addressed this issue using two approaches. First, we examined the effects of valinomycin on transport of C11-sulfonate and laurate in liposomes and in UCP1-containing proteoliposomes with saturating GDP (1 mM) on both sides of the membrane, with the following results (data not shown): K^+ flux increased linearly with concentrations of C11-sulfonate or laurate at constant valinomycin, and a linear

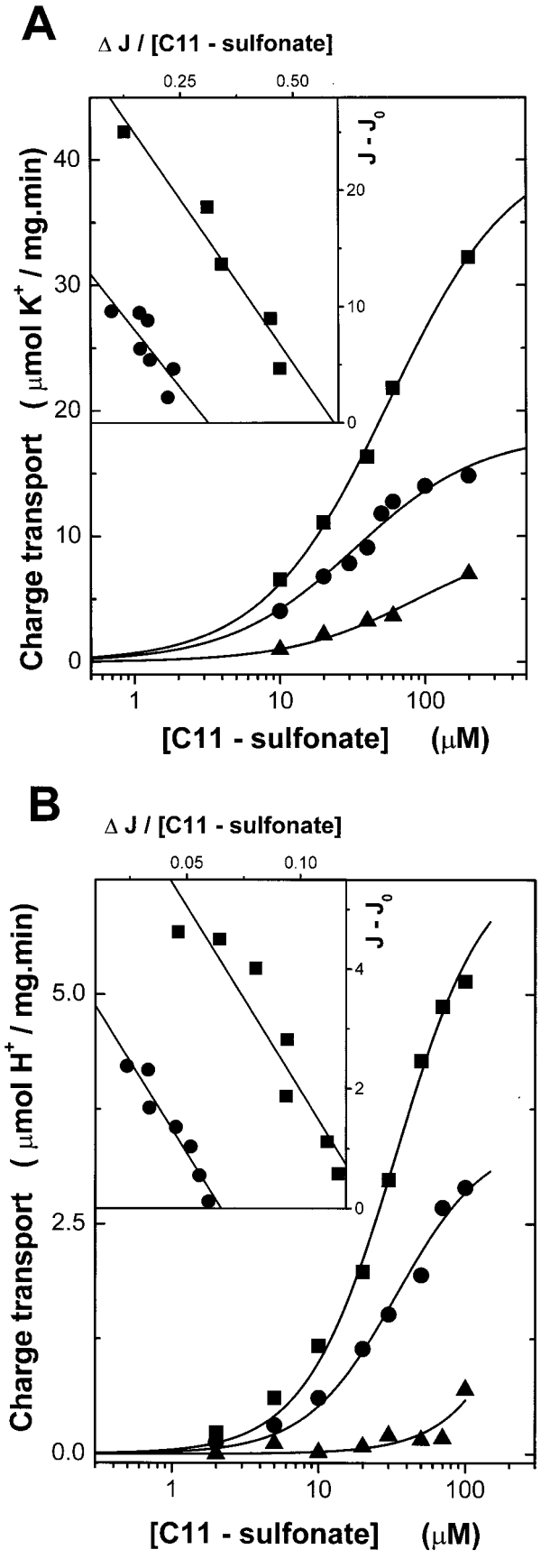

FIG. 4. **UCP1-mediated C11-sulfonate uniport kinetics driven by K^+ diffusion potential (*panel A*) and by pH gradient (*panel B*) in UCP1-containing proteoliposomes.** *Panel A,* concentration dependence of C11-sulfonate influx driven by K^+ gradient plus 100 nM valinomycin. Anion influx was monitored as the compensating K^+ influx (*Charge transport*) by the K^+ selective fluorescence probe PBFI. *Inset,* Eadie-Hofstee plots constructed for the net (differential) fluxes ΔJ, corrected for leak values estimated with 1 mM GDP on both sides of the membrane (▲). In controls with no GDP (■), the apparent K_m was 56 μM, and the V_{max} was 33.3 μmol of K^+ min^{-1} (mg protein)$^{-1}$. In the presence of 1 mM external GDP (●), the apparent K_m was 49 μM, and the V_{max} was 15.3 μmol of K^+ min^{-1} (mg protein)$^{-1}$. *Panel B*, concentration dependence of C11-sulfonate influx driven by pH gradient plus 100 nM CCCP. Anion influx was monitored as the compensating H^+ influx (*Charge transport*) by SPQ quenching. *Inset,* Eadie-Hofstee plots constructed for the net (differential) fluxes ΔJ, when we subtracted the leak values estimated with 1 mM GDP on both sides of the membrane (▲). In controls with no GDP (■), the apparent K_m was 62 μM, and the V_{max} was 8.1 μmol of H^+ min^{-1} (mg protein)$^{-1}$. In the presence of 1 mM external GDP (●), the apparent K_m was 61 μM, and the V_{max} was 4 μmol of H^+ min^{-1} (mg protein)$^{-1}$.

response was also observed when valinomycin was varied. The magnitude of this ion pair transport was about the same with laurate and C11-sulfonate and amounted to less than 10% of the V_{max} of GDP-sensitive transport through UCP. We routinely corrected for this nonspecific leak using the value obtained when saturating GDP was present on both sides of the membrane.

The second approach takes advantage of the fact that there is more than one way to drive anion uniport across the membrane, as diagrammed in Fig. 2. For anion transport driven by a K^+ diffusion gradient, charge transport is measured as K^+ influx (Fig. 2*A*). For anion transport driven by a H^+ diffusion gradient, charge transport is measured as total acid influx (Fig. 2*B*). As shown by the data in Fig. 3, CCCP indeed drives C11-sulfonate uniport across the membrane, and this transport is sensitive to GDP, being maximally inhibited by GDP on both sides of the membrane. CCCP certainly cannot form ion pairs with sulfonate, so this experiment provides new independent evidence that C11-sulfonate is specifically transported by UCP1 (2).

Fig. 4 contains the C11-sulfonate dose dependence and GDP inhibition of charge transport measured as K^+ influx in the valinomycin-K^+ system (Fig. 4*A*), and as H^+ influx in the CCCP-H^+ system (Fig. 4*B*). The results using the two methods are qualitatively the same, including the random orientation of the nucleotide-binding sites, which leads to 50% inhibition by external GDP (2, 17). The V_{max} values in the two assays are different, because the driving forces are different. However, the K_m values are very similar at 41 μM (K^+ gradient) and 49 μM (pH gradient). These results confirm that C11-sulfonate uniport is mediated by reconstituted UCP1 (2).

UCP1-mediated Transport of C11-sulfonate, Cl^-, and H^+ Are Inhibited by GDP with the Same Affinity—We measured the K_i for GDP inhibition of transport at pH 7.2 by reconstituted UCP1 in the presence of 100 μM laurate and 100 μM C11-sulfonate, with the results shown in Fig. 5. The observed K_i values were nearly identical for the two anions: 15.8 ± 2.4 μM (n = 3) and 14.1 ± 2.2 μM (n = 3) for the inhibition of C11-sulfonate and laurate transport, respectively. Moreover, these values are in excellent agreement with values previously obtained for GDP inhibition of Cl^- transport (15–21 μM) (17) and H^+ transport (13–17 μM) (19) through reconstituted UCP1 at pH 7.2.

Because UCP1 is randomly oriented in the vesicles (17), about half of the GDP-binding sites are inaccessible to external GDP. The experiments in Fig. 5 were carried out with 1 mM internal GDP to block these inaccessible sites. We also studied GDP inhibition kinetics in the absence of internal GDP, and the K_i values for both solutes were the same, about 15 μM (data not shown).

C11-sulfonate and Fatty Acids Share a Common Transport Pathway in UCP1—We showed previously that C11-sulfonate

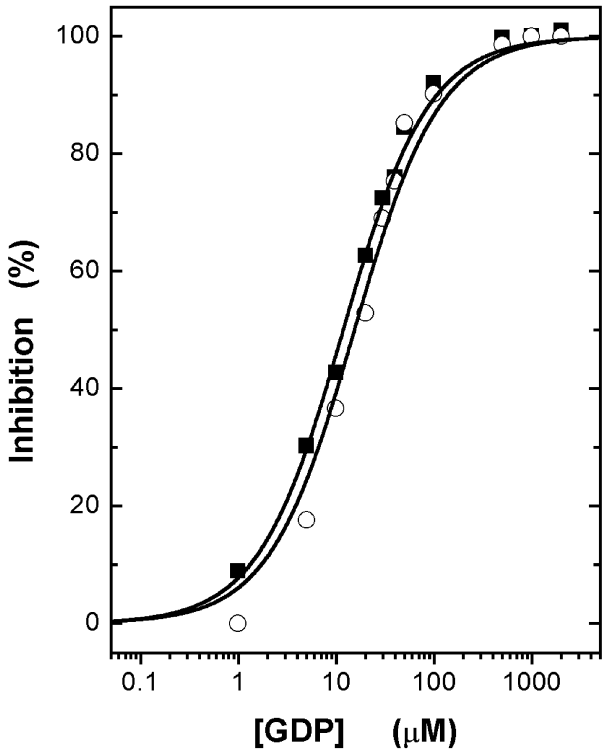

FIG. 5. **The K_i for GDP inhibition of UCP1-mediated laurate and C11-sulfonate transport.** Concentration dependence of GDP inhibition measured in the presence of 100 μM laurate (■) or 100 μM C11-sulfonate (○). Anion influx was driven by a K^+ gradient plus 50 nM valinomycin and monitored as the compensating K^+ influx by the K^+ selective probe PBFI. Proteoliposomes contained 1 mM internal GDP, and 100% inhibition was defined by the flux at external [GDP] = 1 mM. The *solid curve* was plotted by fitting all data to the Hill equation with Hill coefficient = 1. The K_i values obtained in this experiment were 11.8 and 15.3 μM for the inhibition of laurate or C11-sulfonate fluxes, respectively. This experiment is representative of three similar experiments.

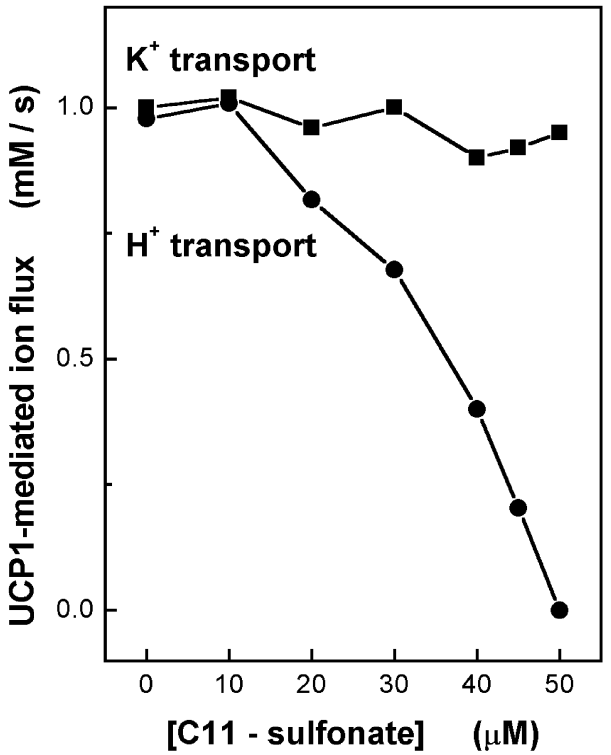

FIG. 6. **Simultaneous lauric acid cycling and C11-sulfonate uniport in UCP1 proteoliposomes.** H^+ efflux and K^+ influx are plotted *versus* C11-sulfonate concentration. Total substrate anion concentration, [C11-sulfonate] + [lauric acid], was maintained constant at 50 μM as [C11-sulfonate] was increased. K^+ influx and H^+ efflux were measured in two parallel preparations of proteoliposomes loaded with PBFI and SPQ, respectively. Ion fluxes were measured in the presence of K^+ gradient as described under "Experimental Procedures."

competitively inhibits laurate-induced H^+ uniport mediated by UCP1 (2). To obtain the data in Fig. 6, we increased C11-sulfonate concentration as total anion concentration (C11-sulfonate plus laurate) was held constant. It can be seen that C11-sulfonate progressively inhibits laurate-dependent H^+ uniport, whereas total charge transport remains constant. This means that laurate and C11-sulfonate are mutually competitive inhibitors and most likely share a common translocation pathway in UCP1 (2).

Ion Pair Transport Converts C11-sulfonate into a Pseudo Fatty Acid Supporting UCP1-mediated H^+ Uniport in Proteoliposomes—Ion-pair transport of amphiphilic amines with amphiphilic anions in mitochondria was first described by Garlid and Nakashima (31). Translocation of the electroneutral ion pair was shown to induce uncoupling or net salt uptake with swelling, depending on experimental conditions. Although ion-pair transport of long-chain alkylsulfonates has not previously been demonstrated, this mechanism would enable the alkylsulfonates to cycle across the membrane, thereby delivering protons and permitting UCP1-mediated uncoupling. The mechanism of this novel uncoupling is described in the legend to Fig. 7.

As shown in Figs. 8 and 9, addition of C11-sulfonate and propranolol catalyzed electrophoretic H^+ flux in liposomes containing UCP1. The traces in Fig. 8*A* show ion pair transport in the bilayer, prior to addition of valinomycin. Propranolol equilibrated rapidly across the membrane, causing internal alkalization due to acid-base equilibration on the interior surface. Upon addition of C11-sulfonate, there is a relatively slow return to the original internal pH, reflecting ion-pair transport of

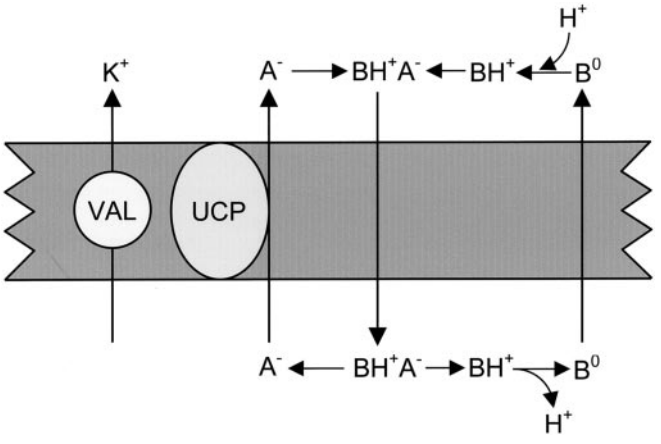

FIG. 7. **Conversion of C11-sulfonate into a pseudo fatty acid by means of ion-pair transport with an amphiphilic amine.** C11-sulfonate cannot flip-flop with protons because of its strong acid character; however, this limitation can be overcome by means of ion pair transport, as depicted in the figure. The protonated amine, BH^+, forms an ion pair with C11-sulfonate, A^-, and the electroneutral complex diffuses across the bilayer. Upon dissociation, the amine loses its proton, and the free base, B^0, diffuses back across the membrane. This part of the cycle occurs in the bilayer and causes C11-sulfonate to distribute and deliver protons as if it were a FA. This step is electroneutral and does not lead to uncoupling. When C11-sulfonate is transported electrophoretically via UCP, a protonophoretic cycle will be set up as shown. Thus, if UCP only transports anions (*e.g.* laurate or C11-sulfonate), then C11-sulfonate should behave like laurate in the presence of an ion-pairing amine.

propranolol-C11-sulfonate to equilibrium. The delivery and consumption of internal acid (mM total acid) by these two steps is given simply by,

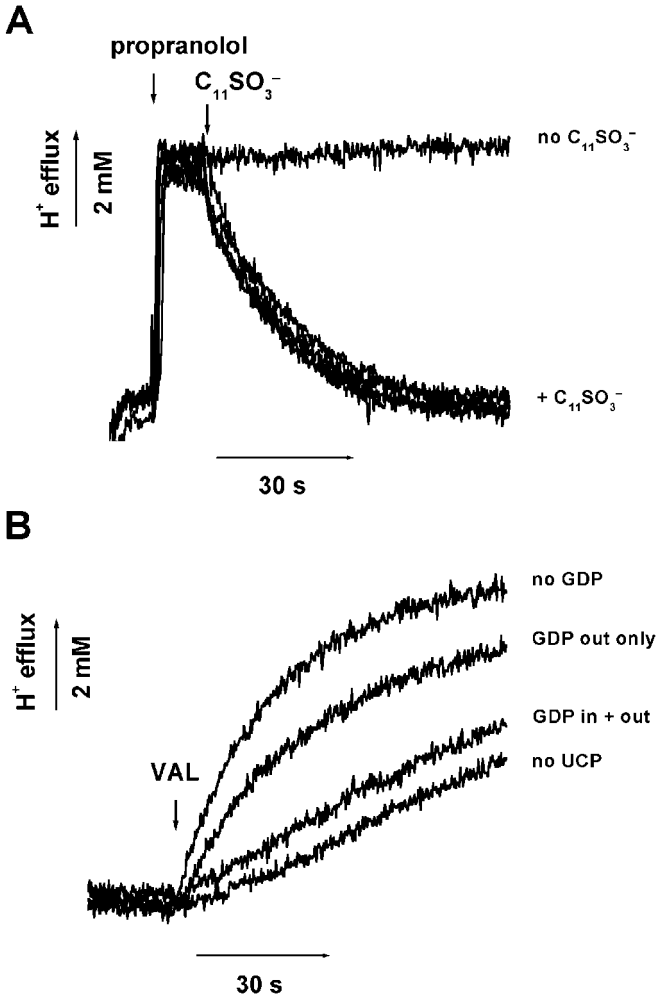

FIG. 8. **C11-sulfonate-dependent H^+ flux in the presence of propranolol in proteoliposomes containing UCP1.** Shown are traces reflecting acid movement across the liposomal membrane as monitored by SPQ fluorescence. *Panel A*, addition of 100 μM propranolol resulted in internal alkalization ("*no $C_{11}SO_3$*"). Further addition of 50 μM C11-sulfonate (+ *$C_{11}SO_3$*) permitted C11-sulfonate to cross the bilayer electroneutrally with protons, as diagrammed in Fig. 7. Four traces with propranolol + $C_{11}SO_3$ are shown. They correspond to the conditions noted in *Panel B*. *Panel B,* upon addition of 100 nM valinomycin (*VAL*) to provide a counterion, UCP1 mediates electrophoretic net H^+ transport. The C11-sulfonate-dependent proton flux was partially inhibited by 1 mM external GDP (*GDP out*) and completely inhibited when GDP was on both sides of the membrane (*GDPin+out*). This inhibited flux was nearly identical to the flux in vesicles lacking UCP1 (*no UCP*). H^+ transport was measured in the presence of a K^+ gradient as described under "Experimental Procedures." This experiment is representative of three similar experiments.

$$\delta[H^+]_i(\text{internal acidification}) = -[BH]_i^+ + [S]_i^- \qquad \text{(Eq. 1)}$$

where $[BH]_i^+$ and $[S]_i^-$ are internalized propranolol and C11-sulfonate, respectively. It is important to note that propranolol exerts its effect on the bilayer and not on UCP1. Thus, these events occur at the same rate and to the same extent independently of GDP or UCP (Fig. 8*A*). Moreover, propranolol had no effect on FA-dependent H^+ flux mediated by UCP (data not shown).

The traces in Fig. 8*B* follow the events that occur after addition of valinomycin, and show that ion pair transport supports GDP-sensitive, electrophoretic proton transport. As described in Fig. 7, C11-sulfonate anion is transported by UCP1; the anion is cycled back with protons through ion pair transport; and the cycle is completed by back-flux and protonation of propranolol base. This experiment provides strong evidence

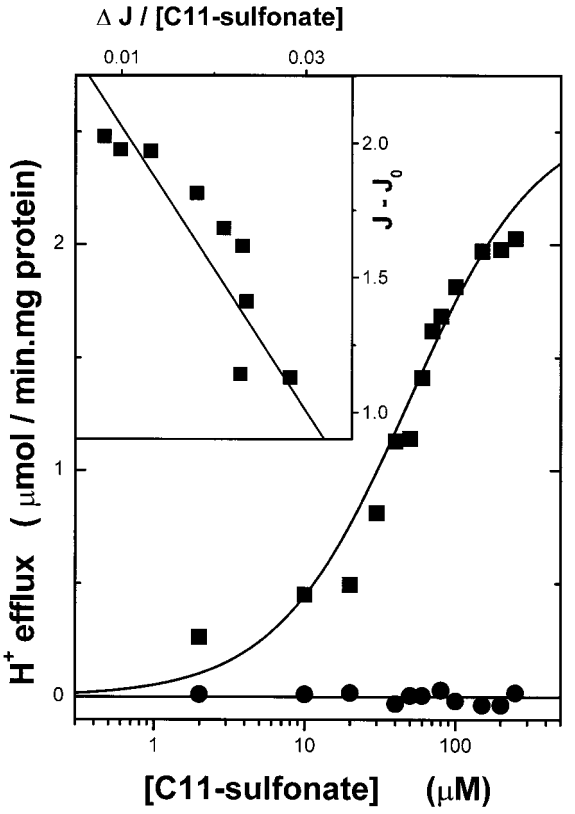

FIG. 9. **Kinetics of C11-sulfonate-dependent H^+ fluxes resulted from ion-pairing with propranolol in proteoliposomes containing UCP1.** Concentration dependence of C11-sulfonate-dependent net H^+ fluxes in the presence (■) or absence (●) of 100 μM propranolol. Propranolol and C11-sulfonate were added to the proteoliposomes in assay medium, followed by addition of 100 nM valinomycin to initiate H^+ influx, as in Fig. 8. The fluxes in the presence of 1 mM GDP inside and outside the proteoliposomes were subtracted. *Inset,* Eadie-Hofstee plot constructed for the net fluxes, ΔJ, yielded K_m 53 μM and V_{max} 2.59 μmol of H^+ min^{-1} (mg protein)$^{-1}$. H^+ transport was measured in the presence of K^+ gradient as described under "Experimental Procedures." This experiment is representative of three similar experiments.

that uncoupling does not require H^+ translocation by UCP1, but rather requires H^+ transport in the bilayer.

Fig. 9 contains the kinetic measurements for UCP1-mediated uncoupling using C11-sulfonate and propranolol. No H^+ flux is detectable in the absence of propranolol, because sulfonates are such strong acids that they cannot be protonated and therefore cannot complete the uncoupling cycle (2). The V_{max} and K_m values obtained for C11-sulfonate were 2.6 μmol of H^+ min^{-1} (mg protein)$^{-1}$ and 48 μM, respectively. The net rate of H^+ transport, after correction for rates observed with saturating GDP on both sides of the membrane, is almost identical with the rate of ion pair transport (compare Fig. 8, *A* and *B*). This indicates that UCP-mediated proton flux is limited by the slow rate of ion pair transport in the bilayer. In the case of unsubstituted FA, FA-dependent H^+ transport is never limited by FA flip-flop in the membrane.

In separate experiments, not shown, we have obtained similar results with C9- and C15-sulfonates, which are analogs of decanoic and palmitic acids, respectively. As previously reported (2, 18), the K_m values decreased with increasing alkyl chain lengths and were 630 and 17 μM for C9- and C15-sulfonate, respectively.

Ion Pair Transport Converts C11-sulfonate into a Pseudo Fatty Acid Supporting Uncoupling of BAT Mitochondria—Fig. 10 contains representative traces of respiration and $\Delta\Psi$ obtained from BAT mitochondria utilizing pyruvate and malate

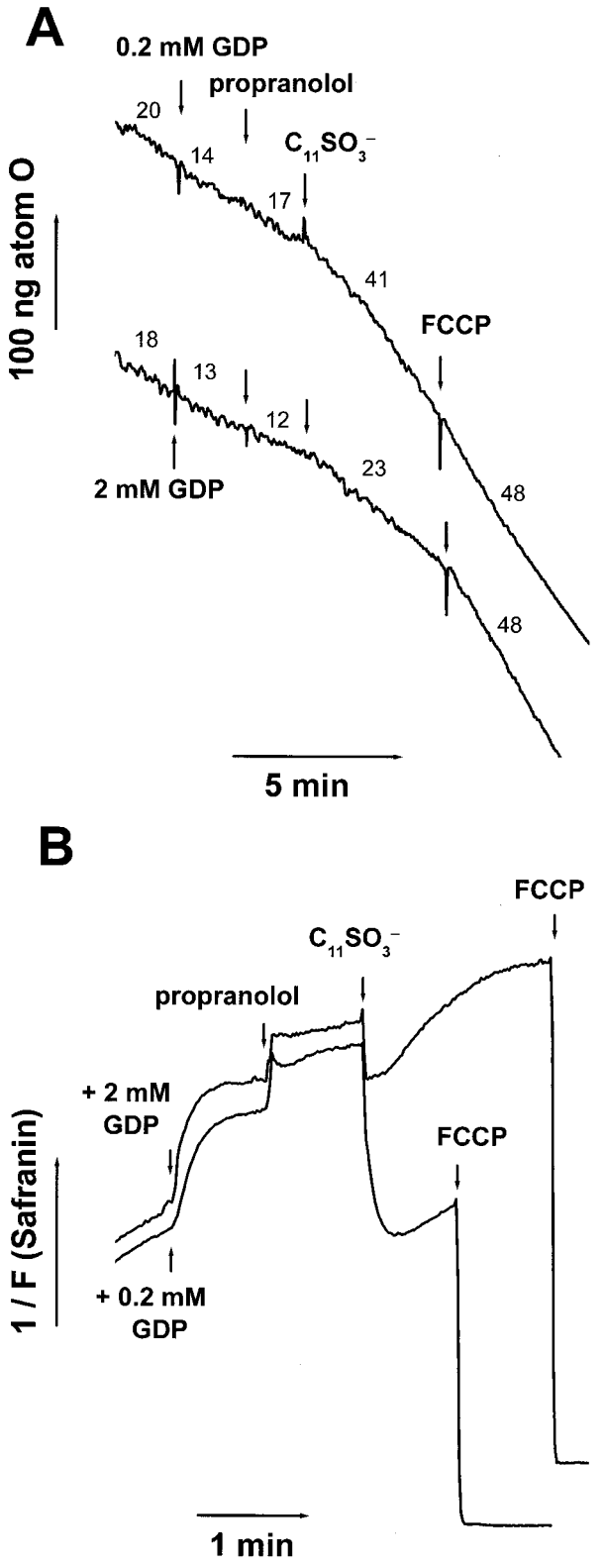

FIG. 10. **Conversion of alkylsulfonates into H^+-translocating cycling substrates in BAT mitochondria.** *Panel A,* respiration traces. The *numbers* on the traces from the Clark polarographic probe indicate respiration rates in nanograms of atoms O min^{-1} (mg protein)$^{-1}$. 0.2 mM GDP was added for the *upper trace*, and 2 mM GDP was added for the *lower trace* (shifted down for clarity). Subsequent additions for both traces were 200 μM propranolol, 200 μM C11-sulfonate ($C_{11}SO_3$), and 2.5 μM FCCP. BAT mitochondria (1 mg/ml) were suspended in medium described under "Experimental Procedures." This experiment is representative of three similar experiments. *Panel B,* membrane potential traces. Inverse Safranin O fluorescence ($1/F$) traces from BAT mitochondrial suspensions (0.5 mg/ml) in media identical to those used for respiratory measurements. 2 mM GDP was added for the *upper trace*, and 0.2 mM GDP was added for the *lower trace*. Subsequent additions for both traces were 200 μM propranolol, 200 μM C11-sulfonate ($C_{11}SO_3$), and 1 μM FCCP. This experiment is representative of three similar experiments.

as substrates. Medium contained 0.2 mM GDP and 2 mM Mg^{2+}. UCP1 was only slightly inhibited under these conditions (Fig. 10*A*), with a corresponding increase in $\Delta\Psi$ (Fig. 10*B*), as previously demonstrated (22, 32). 0.2 mM GDP had only a small effect, presumably because UCP1 is inhibited by free nucleotides (7, 8), which are low in the presence of 2 mM Mg^{2+}. This was verified by demonstrating that 50 μM laurate stimulated respiration and collapsed $\Delta\Psi$ under these conditions (data not shown).

Addition of either 200 μM C11-sulfonate or propranolol did not change the respiration rate. However, when these compounds were both present, respiration increased and $\Delta\Psi$ decreased (Fig. 10), indicating uncoupling. The further observation that ion pair uncoupling was greatly reduced when GDP was increased to 2 mM (Fig. 10, *A* and *B*) shows that the uncoupling was mediated by UCP1. Similar data were obtained when the carnitine cycle was used to deplete endogenous FAs (data not shown).

DISCUSSION

UCP1 has been extensively studied since its discovery nearly 30 years ago (15, 32–34). It is remarkable that the uncoupling mechanism and even the identity of the ionic species transported by UCP1 continue to be a source of controversy and uncertainty in this field (7–14). We will discuss two competing models of UCP transport mechanism, both of which agree that FA are obligatory for uncoupling.

The FA Buffer Model (7–9)—In this model, UCPs transport protons. Intramembrane FA molecules insert their head groups into and along the H^+ transport pathway and provide sites for H^+ translocation. In the latest version (9), FA anions are located along the entire length of UCP; however, the authors insist that the head group is not transported all the way to the other side, despite the presence of a very large electrical driving force. This seems unlikely. Inasmuch as the anionic head group of long-chain alkylsulfonates is transported across the membrane by UCP1, there is no physicochemical basis for exclusion of the FA anionic head group from this pathway.

The FA Protonophore Model (2, 11, 12)—In this model, UCPs transport the anionic FA head group from the inner surface of the inner membrane to the other side. The cycle is completed by rapid, spontaneous flip-flop of protonated FA back across the lipid bilayer, delivering protons.

The FA protonophore model focuses on the known anion transport property of UCP (2, 15–19). We demonstrated Cl^- transport by reconstituted UCP1 (17) and showed that FA inhibit UCP1-mediated Cl^- transport in BAT mitochondria and reconstituted UCP1 (19). We discovered that alkylsulfonates are transported by UCP1 and that the V_{max} and apparent affinity ($1/K_m$) for transport increase with increasing chain length (2, 18). Alkylsulfonates are competitive inhibitors of UCP-mediated Cl^- transport, and the apparent affinity ($1/K_i$) for inhibition also increases with hydrophobicity (18). We infer that the sulfonates interact with a buried hydrophobic site on UCP1 and that access to this site is favored by partitioning in the membrane (18).

Long-chain alkylsulfonates are ideal probes of the UCP transport mechanism for three reasons. (*a*) Except for the head groups, they are identical to FA. (*b*) They mimic FA in their interactions with UCP1: C11-sulfonate is transported by UCP1 with K_m and V_{max} values similar to those obtained for laurate-dependent H^+ transport (2); C11-sulfonate and laurate-

dependent H^+ transport are inhibited by GDP with the same K_i (Fig. 5); and C11-sulfonate and laurate are mutually competitive inhibitors (Fig. 6). (*c*) Alkylsulfonates probe only the UCP-mediated half of the uncoupling cycle, because they cannot flip-flop with protons across the bilayer membrane (2).

Criticisms of the FA Protonophore Model—We will address recent criticisms of the model, beginning with those that focus on the behavior of alkylsulfonates: (*a*) C11-sulfonate inhibition of laurate-dependent H^+ flux is due to competitive removal of FA from the membrane by C11-sulfonate (7, 8). The data in Fig. 1 show clearly that this is not the case. (*b*) C11-sulfonate is not transported by UCP1 but is driven across the membrane in a ternary complex with valinomycin and K^+ (5–8). We show to the contrary that GDP-sensitive C11-sulfonate transport via UCP1 can be driven by a proton gradient and CCCP (Figs. 3 and 4), which excludes the possibility of artificial ternary complex transport of C11-sulfonate. Moreover, ternary complex transport with valinomycin is also observed with laurate, and the magnitude of this transport is small and easily corrected (see "Results"). (*c*) Alkylsulfonate transport has a low sensitivity to GDP, and this sensitivity decreases with increasing chain length (9). This statement is demonstrably incorrect for *long-chain* alkylsulfonates, which are normally studied at concentrations of 100 μM or less. Thus, the K_i values for GDP inhibition of C11-sulfonate and laurate-dependent H^+ transport are the same, as shown in Fig. 5. The value of 15 μM at pH 7.2 is typical of values we have obtained over the years for GDP inhibition of long-chain alkylsulfonate transport, H^+ transport (19), and Cl^- transport (17, 19). The situation is different with *short-chain* alkylsulfonates, which must be studied at high concentrations, because their K_m for transport is high, about 12 mM for hexanesulfonate (2). At 54 mM, K_i values for GDP inhibition increased with chain length from C1 to C6, reaching a maximum of 175 μM for hexanesulfonate (Fig. 9 of Ref. 18). It is established that a variety of anions are weak competitive inhibitors of nucleotide binding to UCP1 (7, 35). Competition by 54 mM sulfonate is not surprising and may be viewed as a weak side reaction of the functional head group occurring only at high concentrations and having nothing to do with transport.

In the first report showing that UCP2 and UCP3 catalyze FA-dependent H^+ transport and C11-sulfonate transport, we suggested that these UCPs also uncouple by the FA protonophore mechanism (4). Klingenberg and Echtay (9) now state that the H^+ flux that we report is "only a few percent of that measured with UCP1." This is incorrect. It is clearly stated in Ref. 4 that the V_{max} values for UCP2 and UCP3 are approximately the same as the V_{max} values observed with UCP1. Moreover, the V_{max} values that we observe with UCP1 are in accord with UCP1 activity in BAT mitochondria (2, 12). On the other hand, H^+ transport rates reported for reconstituted UCP1 by Klingenberg's laboratory are about 90 times higher than are required for uncoupling of BAT mitochondria (7) and are not consistent with what is known about UCP1 activity in BAT mitochondria (12).

It is claimed that β-glucopyranoside palmitate activates H^+ transport although it should not be able to flip through the membrane to deliver protons (9). Actually, this finding appeared only in a review article (7), in which it was stated that the compound is only weakly active. The compound is very unstable, and we suspect that the uncoupling activity is due to the presence of contaminating free palmitate. The finding has no bearing on mechanism until this compound is shown *in the same record* to both activate H^+ transport and be incapable of flip-flop. Sequential FA flip-flop and FA-induced H^+ transport are readily visualized in the same trace (2, 4).

Klingenberg and Echtay (9) raise two interesting points relating Cl^- transport to uncoupling mechanism. (*a*) Cl^- transport is 10-fold slower than H^+ transport and this is inconsistent with FA anion transport by UCP. This is a *non-sequitur,* one cannot predict the rate of Cl^- transport from the rate of laurate transport. Moreover, a proper comparison would be based on V_{max} values, and not on rates. The V_{max} values for Cl^- transport and laurate-induced H^+ transport are 9 and 22 μmol min^{-1} (mg protein)$^{-1}$, respectively (2), so Cl^- transport is 40% of the H^+ transport rate. As we pointed out (2), the V_{max} contains the rate constant for leaving the saturated energy well in the transport site and crossing the second energy barrier. Hydrophilic anions will experience greater difficulty (higher activation energy) in crossing this hydrophobic barrier and will therefore have a lower V_{max}. Thus, the Cl^- data are consistent with our view that Cl^- and other hydrophilic anions are "accidental substrates of the FA anion pathway in UCP" (2). (*b*) A mutation in UCP1 retains H^+ transport but has partially lost Cl^- transport; therefore anion transport capability is not a prerequisite for UCP-mediated H^+ transport (9, 36). It would be more accurate to say that *chloride* transport is not a prerequisite for UCP-mediated H^+ transport. This fact is already known: plant UCP transports alkylsulfonates and FA anions but does not transport Cl^- (3). In UCP1, the K_m for Cl^- is 4 orders of magnitude greater than the K_m for laurate and C11-sulfonate (2), and it is not surprising that some isoforms and mutants have even lower affinity for Cl^-.

Where Does H^+ Transport Occur in UCP-mediated Uncoupling?—According to our model, the sole reason that alkylsulfonates do not support uncoupling is that they cannot deliver protons across the lipid bilayer. It follows that removal of this restriction should result in alkylsulfonate-dependent uncoupling. We were able to remove the restriction through ion pair transport with propranolol, thereby enabling C11-sulfonate to cross the membrane electroneutrally with protons (Fig. 8*A*). Indeed, ion pair transport of C11-sulfonate caused UCP1 to mediate GDP-sensitive, electrophoretic proton flux (Fig. 8*B*). Moreover, propranolol enabled C11-sulfonate to support uncoupling in BAT mitochondria (Fig. 10). This experiment proves that proton transport across the *bilayer* is the only factor preventing UCP-mediated uncoupling by alkylsulfonates, as predicted (2). The available evidence continues to argue against the idea that UCP directly transports protons. Rather, in accord with the FA protonophore model, UCP transports the FA anion, and the protons are translocated by spontaneous FA flip-flop in the bilayer.

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