UCP2-dependent Proton Leak in Isolated Mammalian Mitochondria*

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A role for uncoupling protein (UCP) homologues in mediating the proton leak in mammalian mitochondria is controversial. We subjected insulinoma (INS-1) cells to adenoviral expression of UCP2 or UCP1 and assessed the proton leak as the kinetic relationship between oxygen use and the inner mitochondrial membrane potential. Cells were infected with different amounts of rat UCP2, and, in other experiments, with either UCP2 or UCP1. The relative molar expression of these subtypes was quantified through comparison with histidine-tagged UCP1 or UCP2 proteins engineered by expression in Escherichia coli. Adenoviral infection with UCP2, compared with β-galactosidase, resulted in a dose-dependent shift in kinetics indicating increased H⁺ flux at any given membrane potential. UCP1 also enhanced H⁺ flux, but, on a relative molar basis, the overexpression of the endogenous protein, UCP2, was more potent than UCP1. These results were not due to nonspecific overexpression of mitochondrial protein since UCP1 activity was inhibited by GDP and because overexpression of another membrane carrier protein, the oxoglutarate malate carrier had no effect. UCP2-mediated H⁺ conduc- tion was not GDP sensitive. These data suggest that the UCP homologue, UCP2, mediates the proton leak in mitochondria of a mammalian cell wherein UCP2 is the native subtype.

The issue of whether UCP homologues mediate the proton leak is particularly uncertain for mammalian cells since studies of expressed UCP homologues have largely been carried out in yeast and proteoliposomes (3, 4). In the current studies, we assessed the effect of adenoviral overexpression of UCP2 on the proton leak in mitochondria isolated from rat insulinoma (INS-1) cells, a mammalian cell line in which UCP2 is present as a native protein. To accomplish this, the proton leak was measured as the kinetic relationship between oxygen use and the inner mitochondrial membrane potential under conditions wherein oxygen use is leak-dependent and proportional to H⁺ transport. To our knowledge, these are the first cellular studies to use this approach for assessment of the effect of an overexpressed UCP homologue on leak-dependent H⁺ conductance in mammalian mitochondria.

INS-1 cells were selected for these studies based on prior work in our laboratory (5) demonstrating that UCP2 overexpression enhanced state 4 mitochondrial respiration, decreased the mitochondrial ADP:O ratio (more oxygen required for phosphorylation of a given quantity of ADP), and decreased cell ATP content. Consistent with these results Chan et al. (6, 7) found that adenoviral expression of UCP2 decreased glucose-stimulated insulin release and reduced ATP content in islets of normal rats and reduced membrane potential (rhodamine 123 fluorescence) in βTC6-17 cells, another insulin secreting line. However, neither state 4 respiration nor potential actually measures the proton leak (3). In addition, none of the above studies was controlled for possible nonspecific effects of overexpression of mitochondrial membrane protein per se.

It also uncertain whether catalysis of the proton leak, within a given cell type, is a property restricted to the naturally expressed UCP(s) within that cell type. Of the known UCP subtypes, UCP2 appears to be native to INS-1 cells as well as to normal pancreatic islets (5, 8). Thus, the current studies also examined the relative specificity of UCP2 compared with UCP1 overexpression on the proton leak in INS-1 cells. To quantify and compare the degree of expression of UCP1 and UCP2, we used histidine-tagged UCP1 and UCP2 molecular constructs as standards to obtain an estimate of the relative molar extent of expression of these two UCP subtypes.

In addition, we determined the nucleotide (GDP) sensitivity of the proton leak for these subtypes, an issue that has received little attention in mammalian mitochondria. Beyond the issue of GDP sensitivity per se, we also utilized nucleotide inhibition as a tool for controlling for possible nonspecific effects of overexpressed UCP protein. Finally the issue of nonspecific effects was also addressed through overexpression of another mitochondrial membrane carrier protein, the oxoglutarate malate carrier (OMC) (9).

EXPERIMENTAL PROCEDURES

Reagents and Supplies—PCR primers were obtained through the DNA Core of our Diabetes and Endocrinology Research Center. Affinity...
purified polyclonal rabbit anti-UCP12-A, directed against a 19-amino acid cytoplasmic, COOH-terminal sequence of mouse and rat UCP1 was purchased from Alpha Diagnostics International (San Antonio, TX). Affinity purified goat anti-UCP2, directed against the identical amino termini of mouse and rat UCP2, was purchased from Research Diagnostics (Flanders, NJ). The specific antibodies were raised from the same manufacturers. Antibody against the OMC was obtained from rabbits immunized with two polypeptides prepared from conserved regions of OMC sequences (Arg1-Ser9 and Asp28-Gly37) and characterized in previous studies (10, 11). Mouse antibody against the six-residue histidine tag, His-probe (FL) was purchased from Santa Cruz Biology, Inc. (Santa Cruz, CA). Other reagents, kits, and supplies were as specified or purchased from standard sources.

Adenovirus Generation—The cDNA encoding the full-length rat UCP2 was amplified from isolated spleen total RNA by reverse transcriptase-PCR according to standard methodology (12), ligated into the shuttle plasmid pAd5CMV-Neo, and, grown and purified by the Gene Transfer Vector Core of our institution as we previously described (5). Adenoviral UCP1 was engineered by the laboratory of Dr. Michael Brownlee (13) at the Albert Einstein College of Medicine using the same vector. pAd5CMV-Neo, expressing bacterial β-galactosidase rather than UCP1 or UCP2 (3) was obtained from our Gene Transfer Vector Core for use as control.

The sense and antisense primers used for constructing the full-length rat OMC clone were: 5'-AGGGCCATCTAAGAGGTGGCGATGGCGGGCAGA-3' and 5'-TCAGCCATGAGAGAGACGCT-3' (positions 1–10 and 943–965, Genbank™ accession number U84727), respectively. The sense primer included an appended Kozak sequence to enhance transcription of the product (14). The resulting PCR product was ligated into pCR II (Invitrogen, Carlsbad, CA) and transformed into DH5α cells. Following purification of DNA, the full-length OMC clone was excised using a flanking EcoRI site and ligated into the EcoRI restriction site in the shuttle vector pAd5CMV-Neo.

Cell Culture Adenoviral Infection—INS-1 cells were kindly provided by Dr. Claus Wolheim (Geneva, Switzerland). Cells were seeded to 100-mm dishes for mitochondrial isolation and 12-well plates for lipid and glucose oxidation. Cells were grown in RPMI with 10% fetal bovine serum. Cells were grown in RPMI with 10% fetal bovine serum and incubated at 37 °C in 5% CO2. Cells were allowed to reach confluence. In previous work, we described the efficacy of infection for varying viral loads as determined by staining for β-galactosidase (5).

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Mitochondrial Isolation—Cells were washed with phosphate-buffered saline and scraped. Collected cells were homogenized using a Dounce homogenizer in ice-cold homogenization buffer (0.25 M sucrose, 50 mM Tris, 0.1 mM EDTA, pH 7.2) with 0.1% fatty acid-free bovine serum albumin. Isolated mitochondria (0.7 mg mitochondrial protein), as an ionophore, was included so the Rb cation could enter mitochondria. It is important to note (Table I) that there was little difference between matrix volume or binding correction between the mitochondrial groups studied so any error in these measurements would have minimal effect on the comparative studies of membrane potential. Furthermore, due to the logarithmic nature of the Nernst equation, volume and binding differences actually alter Δψ relatively little.

Determination of the Proton Leak—The proton leak was assessed as the relationship of H+ flux to mitochondrial membrane potential through simultaneous recording of oxygen consumption and potential. Mitochondria were respiring in a respiration chamber in respiratory media (220 mM mannitol, 70 mM sucrose, 2.5 mM KH2PO4, 2 mM MgCl2, 1 mM EDTA, 2 mM HEPES, pH 7.4) with 0.1% fatty acid-free bovine serum albumin. The homogenate was centrifuged at 500 × g for 10 min. The pellet was discarded and the supernatant was centrifuged again at 10,000 × g for 10 min to obtain the mitochondrial pellet. The resulting pellet was then washed three times in homogenization buffer without bovine serum albumin and resuspended in respiration media described below.

For immunoblotting, mitochondria were washed as above and homogenized by solubilization in detergent RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA). Protein was determined by the Bradford method using a dye reagent purchased from Bio-Rad.

Polarography—Mitochondrial respiration was measured using a Clark miniature oxygen electrode and small (0.6 ml) volume chamber with stir bar (Instech Laboratories, Inc., Plymouth Meeting, PA) at 37 °C in respiratory media (220 mM mannitol, 70 mM sucrose, 2.5 mM KH2PO4, 2 mM MgCl2, 1 mM EDTA, 2 mM HEPES, pH 7.4) with 0.1% fatty acid-free bovine serum albumin. Isolated mitochondria (0.7–1.0 mg of protein/ml) were incubated in the respiratory media and oxygen consumption quantified under the conditions indicated.

Membrane Potential—Membrane potential was calculated in millivolts as described (15) from the concentrations inside (Ci) and outside (Co) the mitochondrial matrix of the lipophilic cation tetraphenyl phosphonium (TPP+). If the Nernst equation, Δψ = RT ln F, where z = valence of cation, T and F are standard thermodynamic quantities (R designates the gas constant or 8.314 joules/K, T the temperature, and F represents Faraday’s constant or 96,494 cal/mol). So, at a temperature of 310 degrees K (37 °C) at one atmosphere for and a univalent cation, Δψ = 61.5 log (Ci/Co). TPP+ concentrations external to the mitochondria were determined by an electrode sensitive to this cation constructed according to the design described by Kamo et al. (15). The sensor membrane was constructed of a 2-mm Duran™ reference electrode (DRIREF-2SH, World Precision Instruments, Inc., Sarasota, FL) and a 2-mm Ag/AgCl wire. The potential difference between this electrode and a 2-mm Dri-Ref™ reference electrode (DRIREF-2SH, World Precision Instruments, Inc., Sarasota, FL) was determined using a potentiometer (mV setting available on a pH standard meter). Both the TPP+ and reference electrodes were inserted into the Clarke chamber through ports drilled by the University of Iowa Biomedical Engineering Department so that membrane potential and oxygen consumption could be recorded simultaneously. Electrode potential was highly sensitive to added TPP+ concentrations ranging from 10-10 to 10-2 on a log scale and nearly linear from 10-6 to 10-2. The electrode was not sensitive to other cations including calcium and magnesium.

C, for TPP+ was determined during mitochondrial incubations from the recorded electrode potential based on prior calibration of the electrode as a function of TPP+ added in the absence of mitochondria. C, is determined from the known amount of TPP+ added (total) and external concentrations. To determine M, it was also necessary to know mitochondrial matrix volume and the extent to which TPP+ is bound to mitochondrial protein as opposed to freely present within the matrix. Mitochondrial matrix volumes under the conditions of incubation were determined from exclusion volumes of [3H]H2O and [14C]sucrose (16) for mitochondria isolated from INS-1 cells infected with viral titers (3.1 × 109) of UCP1 (with and without 1 mM GDP), UCP2, and β-galactosidase. The binding correction was determined by the difference in measured [3H]TPP+ and [3919]<14C>Rb+ accumulation (17). Valinomycin (320 pmol/mg of mitochondrial protein), as an ionophore, was included so the Rb cation could enter mitochondria. It is important to note (Table I) that there was little difference between matrix volume or binding correction between the mitochondrial groups studied so any error in these measurements would have minimal effect on the comparative studies of membrane potential. Furthermore, due to the logarithmic nature of the Nernst equation, volume and binding differences actually alter △ψ relatively little.
UCP2 expression in INS-1 mitochondria, we engineered UCP1 and UCP2 proteins tagged with 6-histidine residues at the amino termini. This was accomplished using a commercially available expression and purification system (QiAexpress, Qiagen). We used standard PCR methodology (12) to clone the insert (coding for the particular UCP) with appropriate 5’ and 3’ extensions into pQE-60 (Qiagen) for bacterial expression. Upstream primers included long (98 bases) sense oligonucleotides encoding (in 5’ to 3’ order) the appropriate restriction site, a ribosomal binding site, ATG, the His6 tag, an enterokinase site, and 22 or 21 residues of the 5’ end of the coding region of UCP1 or UCP2, respectively. Antisense oligonucleotides downstream encoding the downstream restriction site, stop codon, and 18 residues of the 3’ end of the coding region of UCP1 or UCP2. Plasmids (pQE-60) with inserts were verified by fluorescent DNA sequencing by the DNA core of our Diabetes and Endocrinology Research Center.

To generate the expressed proteins, M15 Escherichia coli were transformed with pQE-60 containing the UCP1 or UCP2 insert and grown to $A_{	ext{660}} = 0.6$. Isopropyl-$\beta$-thiogalactopyranoside, 1 $\text{mM}$, was added to induce expression. Cells were grown in 2-liter flasks and centrifuged at 5,000 × $g$ for 15 min. Cells were lysed in B-PER reagent (Pierce, Inc.) for 15 min at room temperature, centrifuged at 25,000 × $g$ for 15 min, and suspended in B-PER containing lysyoo, 200 $\mu$g/ml. After 15 min the lysate was diluted with an equal volume of 1:10 B-PER and centrifuged at 25,000 × $g$, 15 min. The inclusion bodies were washed three times in 150 mM potassium phosphate, 25 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, pH 7.8, and solubilized in 1.5% Sarkosyl.

These bacterial extracts were carefully quantified for protein by triplicate determination using both the Bradford and Lowry techniques (which differed by <5%). The histidine tag immunoreactivity of the tagged UCP1 and UCP2 proteins were then determined by immunoblot analysis. In this way, the relative histidine tag immunoreactivity (“histidine units”) per microgram of protein were determined and used as a measure of the relative molar content of UCP1 or UCP2. The extracts were then employed as standards run in multiple dilutions for quantitative immunoblotting to determine the content of UCP1 or UCP2 in histidine units in extracts of the mitochondria employed in the electrochemical measurement of the kinetics of the proton leak.

RESULTS

Mitochondrial matrix volumes and the TPP$^+$ binding correction were determined for mitochondria expressing $\beta$-galactosidase, UCP1 and UCP2 after exposures to viral titers of 3.1 × 10$^6$ plaque-forming units (pfu)/cm$^2$ or UCP1 with added GDP (Table I). TPP$^+$ binding was similar for all conditions studied, so an overall mean value was used for correction of the matrix TPP$^+$ concentrations. Likewise, UCP1 and UCP2 protein expression had little effect on matrix volume. Since, there was a slight (nonsignificant) increase in the volumes for mitochondria expressing the uncoupling proteins, the mean determined volumes (for each viral exposure) were used for calculation of membrane potentials for mitochondria from cells expressing the listed proteins. However, using an overall mean value for volume for all conditions studied would have had negligible effects on the data. Thus, for experiments involving UCP2 titers less than 3.1 × 10$^6$ pfu/cm$^2$, matrix volumes used in the calculations were as indicated for the higher titers.

H$^+$ flux as a function of membrane potential was determined in mitochondria isolated from INS-1 cells infected with different viral titers of adenoviral UCP2 or $\beta$-galactosidase (Fig. 1).

Individual data points represent values for both membrane potential and H$^+$ flux for a given amount of added malonate. Thus, error bars represent variation for each measured parameter and are directed along both the x and y axes. Adenoviral infection with UCP2, compared with $\beta$-galactosidase, resulted in a dose (viral load)-dependent shift upward and to the left indicating increased H$^+$ flux at any given membrane potential.

To better quantify the degree of UCP2 overexpression compared with control, mitochondria from each of the preparations studied were assayed for UCP2 expression by immunoblot analysis. The signal densities were normalized to a dilution series of a standard pooled sample (prepared from mitochondria of a large pool of INS-1 cells infected with adenoviral UCP2) included on each blot. A representative blot indicating the level of expression of UCP2 at each of the viral titers is shown in Fig. 2. Fig. 2 also shows the quantitative data over three repetitions of this experiment indicating the relative level of overexpression for each of the viral titers.

For statistical assessment of the effects of UCP2 to alter potential and proton flux, the data in Fig. 1 were subject to polynomial curve fitting. Each individual curve for each of the viral doses was used to determine the potential at a H$^+$ flux of 90 nmol of H$^+$/min/mg. This value of 90, although arbitrary, represents H$^+$ flux at approximately one-half of the highest (not necessarily maximal) rate recorded (for the highest degree of expressed UCP2) and falls within a range where potential could be assessed at each viral titer or level of UCP expression.

Fig. 3, panel A, shows that membrane potential at constant proton flux (90 nmol of H$^+$/min/mg) correlated negatively to flux at any given membrane potential. A value of 160 mV was selected since this value falls in a range where flux could be assessed for the various levels of UCP2 expression (Fig. 1). Fig. 3, panel B, shows that proton flux at this constant potential correlated positively to UCP2 expression ($p = 0.002$).

We further examined leak-dependent H$^+$ conductance in mitochondria of INS-1 cells as affected by adenoviral overexpression of UCP1, UCP2, and $\beta$-galactosidase (Fig. 4). For each of these conditions, mitochondria from the same preparation were studied in the presence or absence of 1 mM GDP, a known inhibitor of UCP1-mediated H$^+$ conduction (1, 2). As in Fig. 1, oxygen use and potential were determined in mitochondria respiring on succinate in the presence of oligomycin, rotenone, and nigericin. Adenoviral infection with UCP2 or UCP1 compared with $\beta$-galactosidase resulted in a marked shift upward and to the left indicating increased H$^+$ flux at any given membrane potential. GDP shifted the curve for UCP1 almost back to the position of $\beta$-galactosidase but did not alter the effect of UCP2 overexpression. There did appear to be a slight shift for $\beta$-galactosidase, but, this was considerably less than for UCP1, especially at the higher values of H$^+$ flux wherein the S.E. for potential was considerably less.

To obtain standards for quantitative comparison of the relative molar amounts of UCP1 and UCP2 expressed by the mitochondria in Fig. 4, we generated histidine-tagged UCP1 and UCP2 by expression in E. coli. We then compared the relative level of immunoreactive histidine tag expression of these bacterial extracts (Fig. 5, panel A) and plotted density against micrograms of inclusion body protein (Fig. 5, panel B). We arbitrarily assigned a histidine tag content (histidine units per micrograms of protein) of 100 for the UCP1 preparation. By comparison of the slopes (Fig. 5, panel B), the UCP2 preparation contains 770 histidine units per $\mu$g. Note that when the band densities of Fig. 5, panel B, are plotted against micrograms of protein on a log scale (Fig. 5, panel C) the curves are

| Condition (viral protein expressed) | Matrix volume (µg/mg mitochondrial protein) | TPP$^+$ binding correction |
|-----------------------------------|-------------------------------------------|---------------------------|
| $\beta$-Galactosidase              | 0.94 ± 0.07 (n = 4)                        | 0.29 ± 0.04 (n = 4)       |
| UCP2                              | 1.06 ± 0.22 (n = 5)                        | 0.38 ± 0.04 (n = 6)       |
| UCP1                              | 1.18 ± 0.22 (n = 4)                        | 0.36 ± 0.07 (n = 7)       |
| UCP1 (presence of 1 mM GDP)       | 1.27 ± 0.22 (n = 4)                        | 0.33 ± 0.07 (n = 7)       |
roughly parallel supporting the specificity of the antibody for the His$_6$ epitope.

We then compared UCP2 immunoreactivity of the INS-1 mitochondria overexpressing UCP2 in Fig. 4 with histidine-tagged UCP2 (Fig. 6, panel A) and, likewise, the UCP1 immunoreactivity of the INS-1 mitochondria expressing UCP1 in Fig. 4 with histidine-tagged UCP1 (Fig. 6, panel B). We were thus able to calculate the expression of UCP1 and UCP2 (for the mitochondria) of Fig. 4 in histidine units per $\mu$g of mitochondrial protein (Fig. 6). From knowledge of the number of micrograms of mitochondrial protein added to the respiratory chambers in the experiments of Fig. 4, we calculated the number of histidine units of UCP1 or UCP2 added for each run. In this way, we calculated that the mean amount of UCP2 within the mitochondria used in the studies of Fig. 4 was $3.6 \pm 0.2 \times 10^3$ histidine units (mean + S.E.) per chamber and the amount of UCP1 was $5.8 \pm 1.2 \times 10^3$ histidine units per chamber. Thus, UCP2 shifted the curve of $H^+$ flux versus potential further to the left and upwards than UCP1 (Fig. 4) even though the molar amounts of UCP2 expressed were less than UCP1.

To quantify the expression of UCP2 in the mitochondrial samples of Figs. 1 and 2, the standard pool samples of Fig. 2 were compared with histidine-tagged UCP by immunoblotting (not shown) with the result that the standard pool contained $12.4$ histidine units/$\mu$g of protein. Hence, the arbitrary units in Fig. 2 can be converted to histidine units. From these results,
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FIG. 3. Panel A, inner membrane potential at equivalent respiration (15 nmol of O/min/mg or 90 nmol of H⁺/min/mg) of mitochondria isolated from cells exposed to different viral loads and expressing different amounts of UCP2 (see Fig. 2). Panel B, H⁺ flux at constant potential (180 mV) for the same mitochondrial preparations. Data were analyzed by 2-tailed Pearson correlation.

FIG. 4. H⁺ flux in the presence or absence of 1 mM GDP as a function of membrane potential in mitochondria isolated from INS-1 cells infected with the indicated adenoviral constructs. Data represent mean ± S.E., n = 4 for each point.

As evident from Fig. 7, expression of the OMC protein had no effect on proton leak-dependent H⁺ conductance. Since matrix volumes and TPP⁺ binding differed little for expression of UCP2, UCP1, and β-galactosidase (Table I), a matrix volume of 1.0 µm⁴/mg and the same TPP⁺ binding correction used for UCP2, UCP1, and β-galactosidase were used for these calculations. As discussed under “Experimental Procedures,” these are robust assumptions given the logarithmic nature of the calculations.

DISCUSSION

UCP1, originally termed thermogenin, appears to play a major role in cold adaptation and serves as a source of heat in hibernating animals. There is now considerable evidence that this occurs through conductance of protons from the outside to inside (matrix space) of the inner mitochondrial membrane in brown fat, thereby, dissipating the electrochemical gradient. This is supported by expression in yeast (24–27) and reconstitution in liposomes (25) as well as in studies of BAT mitochondria (24). A role(s) for UCP homologues to dissipate energy has likewise been proposed, not only for heat production, but to serve other purposes as well such as reducing the mitochondrial generation of reactive oxygen radicals or regulating the metabolism of free fatty acids (28).

However, independent of the physiologic purpose(s) of UCP homologues, the fundamental question remains as to whether or not these proteins actually catalyze H⁺ transport (3). Major support for a role of UCP2 and/or UCP3 in H⁺ conductance includes sequence homology to UCP1, decreased ability of yeast expressing UCP2, UCP1, and β-galactosidase (Table I), a matrix volume of 1.0 µm⁴/mg and the same TPP⁺ binding correction used for UCP2, UCP1, and β-galactosidase were used for these calculations. As discussed under “Experimental Procedures,” these are robust assumptions given the logarithmic nature of the calculations.

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UCP3 may couple yeast mitochondria, there is evidence that this may be secondary to nonspecific impairment of mitochondrial integrity rather than a specific uncoupling action of the inserted protein (37). The effect of UCP1 to increase H\(^{+}\) conductance can be confirmed in the sense that it is sensitive to inhibition by GDP (3), but, no similar means of assessment exists for UCP2 or UCP3. In this regard, studies of UCP homologue expression in yeast show that they are not nucleotide sensitive (37–40). Also, physiologic data raise questions as to the role of UCP homologues in H\(^{+}\) conductance. Cadenas et al. (41) found that leak-dependent H\(^{+}\) conductance in mitochondria isolated from skeletal muscle of fasted rats remained unchanged despite a 4–5-fold increase in both UCP2 and UCP3.
mRNA and a 2-fold increase in UCP3 protein levels. In addition, UCP2 message increased severalfold in BAT from UCP1 knockout mice. However, resultant properties of uncoupling were not observed (40).

Our data support the concept that UCP2, like UCP1, does catalyze the proton leak of the inner mitochondrial membrane when studied in a mammalian cell wherein UCP2 is the predominant native uncoupling protein. We show that both UCP1 and UCP2 overexpression in mitochondria isolated from INS-1 cells enhance the rate of oxygen consumption at any given magnitude of membrane potential using a kinetic approach, which is considered the most reliable means of assessing the proton leak (3, 19).

Our observations suggest that overexpression in INS-1 mitochondria is dose (degree of UCP2 expression)-dependent. Although UCP2 content was not quantified in absolute molar terms, enhanced \( H^+ \) conductance was demonstrable at levels \( \sim 3-4 \) fold above native expression (Figs. 1 and 2). At this lower level of overexpression, there was still a substantial shift in the kinetics of \( H^+ \) flux versus potential (Fig. 1), suggesting that this degree of expression does in fact enhance the proton leak. This extent of enhanced expression seems physiologically feasible based on observations of a 4-14-fold increases in UCP2 mRNA observed in muscle of insulin-deficient diabetic rats (42), in fat and pancreatic islet tissues of rats given leptin (43), or in BAT in mice treated with a \( \beta_3 \)-adrenergic agonist (44) or subject to UCP1 ablation (40).

As shown in Fig. 3, at a constant membrane potential of 160 mV, \( H^+ \) flux was significantly correlated with UCP2 expression. A linear relationship between these parameters would be expected if the amount of UCP2 protein were rate-limiting. However, we point out that no firm conclusion can be drawn about the implications of this relationship since correlation does not imply actual linearity.

GDP shifted the curve for UCP1 (Fig. 4) almost back to the position for \( \beta \)-galactosidase. This is consistent with the well-documented effect of GDP to inhibit UCP1-mediated \( H^+ \) conduction in reconstituted systems and in BAT mitochondria in which UCP1 is abundant (45, 46). The lack of inhibition by GDP of UCP2 is as expected based on prior studies in yeast and in BAT of UCP1-deficient mice (38, 39, 47) suggesting that UCP homologues are insensitive to GDP. There does appear to be a slight shift for \( \beta \)-galactosidase, perhaps reflecting inhibition of native uncoupling. But, this is considerably less than for UCP1, especially at the higher values of \( H^+ \) flux wherein the S.E. for potential is considerably less.

Stuart et al. (37) performed quantitative immunoblot analysis of expressed UCP2 and recently reported that UCP2 in yeast mitochondria did not uncouple oxidative phosphorylation unless present at levels an order of magnitude above those seen physiologically. At that level, these investigators did observe a left shift in the kinetics of \( H^+ \) flux versus potential but attributed the change to artifact likely from impaired mitochondrial integrity.

However, our current results suggest that UCP-mediated uncoupling in INS-1 mitochondria is not due to nonspecific effects of mitochondrial protein overexpression or impaired mitochondrial integrity. The inhibition of UCP1-mediated \( H^+ \) conduction by GDP (Fig. 4) implies that the effect of UCP1 is not simply a result of nonspecific effects of protein overexpression and there is no known reason why GDP should improve any impaired mitochondrial integrity. Also, for an equivalent extent of uncoupling (compare Figs. 1 and 4), UCP2 was more potent. Thus, if UCP1-mediated conduction is not due to nonspecific effects of protein insertion, it seems unlikely that that mediated by a lower molar content of UCP2 should be the result of this phenomena. Furthermore, this issue was also addressed by expression of another mitochondrial inner membrane carrier (9) of similar molecular size, the OMC protein. This resulted in no change in the kinetics of \( H^+ \) flux versus membrane potential (Fig. 7), consistent with a lack of nonspecific effects to induce a proton leak. Although not quantified in molar terms, the expression of the OMC appeared substantial (Fig. 7) and, using the same adenoviral backbone as that for UCP2 and UCP1, the cells were exposed to OMC viral particles at a titer equivalent to the highest used for UCP2 (Figs. 1 and 2).

Our data suggest that, on a molar basis, when overexpressed in INS-1 mitochondria, UCP2 is more effective in catalyzing the proton leak than UCP1. The reason for this is unclear. However, of the known UCPs, UCP2 appears to be the major protein expressed in INS-1 as well as normal rat pancreatic islets (5, 8). So, we can speculate that, at the molecular level, overexpressed UCP2 might be more effectively situated and, thus, functionally more active compared with UCP1.

Prior studies of adenoviral expression of UCP2 in normal pancreatic islets demonstrated decreased ATP, increased fat metabolism, and impaired insulin release (6, 7). On the other hand, when expressed in islets of obese ZDF rats, UCP2 actually improved insulin secretion (8), but, this was likely the result of a reduction in the large accumulation of \( \beta \)-cell fat and decreased lipotoxicity. Our past studies of UCP2 overexpression in INS-1 cells demonstrated enhanced mitochondrial respiration, reduced cellular ATP, increased fat oxidation, and reduced insulin release (5). However, none of the above studies actually examined the proton leak and none were controlled for nonspecific effects of overexpressed mitochondrial protein, since control viral exposures did not express a mitochondrial membrane protein or examine the quantitative extent of overexpression. Our past studies of INS-1 cells (5) did use an adenoviral MnSOD protein as control but the protein is, to a large extent, expressed in the mitochondrial matrix rather than the inner membrane per se. Hence, the current findings now provide evidence that the above mentioned alterations in islet cell metabolism and insulin release are associated with and may be the direct result of a UCP2-regulated mitochondrial proton leak.

In summary, UCP2 increases \( H^+ \) flux in a dose-dependent fashion over a range of membrane potentials in mitochondria of INS-1 cells. On a molar basis, UCP2 has a higher conductance than UCP1 in these mitochondria. However, the \( H^+ \) conductance of UCP2, unlike UCP1, is nucleotide insensitive. Thus, the UCP homologue, UCP2, appears to enhance leak-dependent \( H^+ \) conductance in mitochondria of a mammalian cell line wherein this subtype is the native or endogenously expressed uncoupling protein.

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