Chloride Channel Properties of the Uncoupling Protein from Brown Adipose Tissue Mitochondria: A Patch-Clamp Study†

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ABSTRACT: The uncoupling protein (UCP) from brown adipose tissue mitochondria possesses H^+ and Cl^- transport activities [reviewed in Klingenberg, M. (1990) *Trends Biochem. Sci. 15,* 108−112]. Being a member of a mitochondrial carrier family, the transport of H^+ and Cl^- is carrier-like, i.e., much slower as compared to channels. Here we report that UCP reconstituted into giant liposomes displays stable chloride channel properties under patch-clamp conditions. The transport inhibitors (GTP, GDP, ATP, and ADP) also inhibit this channel in a reversible way, showing that the channel activity is associated with UCP. The slightly inward-rectifying chloride channel has a unit conductance of ∼75 pS in symmetrical 100 mM KCl and closes at high positive potentials on the matrix side of UCP. Channel gatings switch from slow open−closure transitions to fast flickerings as the holding potential increases over +60 mV. Substitution experiments reveal a strong discrimination against cations [$P(Cl^-)/P(K^+) \approx 17$] and a permeability ratio order of $Cl^- > Br^- > F^- > SCN^- > I^- > NO_3^- > SO_4^{2-} > HPO_4^{2-} >$ gluconate. Nucleotide inhibition studies indicate that 70% UCP molecules had its matrix side oriented outside in the giant liposomes. Fatty acids, pH, divalent cations (Ca^{2+} and Mg^{2+}), and mersalyl do not influence these Cl^- currents. The Cl^- channel can be blocked by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) from the matrix side of UCP. The data are consistent with a dimer consisting of two monomeric 75-pS Cl^- channels or with a monomeric 150-pS channel having a 50% subconductance state. The channel current increases with Cl^- concentration showing a typical saturation curve with $K_m \approx 63$ mM and $g_{max} \approx 120$ pS (100 mM KCl in the pipet). The Cl^- conductance measured under these conditions is 6 orders of magnitude higher than the Cl^- transport activity reported earlier, suggesting that the UCP has the potential of behaving as an anion channel.

A permeability for chloride has long been known to be an integral property of the uncoupling protein (UCP)[1] from the brown adipose tissue mitochondria (Nicholls & Lindberg, 1973; Nicholls, 1979; Jezek et al., 1990). This 33-kDa protein from the inner mitochondrial membrane acts primarily as a H^+ carrier [for a review, see Nicholls and Locke (1984) and Klingenberg (1990)], a function that has been related to nonshivering thermogenesis of this specialized tissue in mammals (Smith & Horwitz, 1969; Nicholls, 1974; Himms-Hagen, 1976). Since the first demonstration in swelling experiments by Nicholls and Lindberg (1973), the Cl^- transport activity was also confirmed later in vesicles reconstituted with the isolated protein (Jezek et al., 1990). Thus, UCP possesses a low GDP-sensitive Cl^- transport activity (turnover number of 0.1−10 ions per second) with a weak pH dependence and an anion selectivity of $I^- > Br^- > Cl^-$.

In view of a recent demonstration of channel-type Cl^- conductance in the phosphate/triosephosphate exchanger from chloroplasts (Schwarz et al., 1994) and our own interest in exploring other methods to examine the transport modes of UCP, we embarked on patch-clamp studies of the UCP. Since the UCP is located in the inner mitochondrial membrane, it is difficult to obtain patches on the small mitoplast, let alone the presence of other channels which might interfere in the inner membrane (Tedeschi & Kinnally, 1994; Klitsch & Siemen, 1991). The giant liposome approach makes possible a patch-clamp study of ion channels in the reconstituted system (Criado & Keller, 1987; Keller et al., 1988). According to this procedure, cell-size "giant" liposomes containing the ion channel of interest can be generated by a dehydration−rehydration treatment of small proteoliposomes, thus allowing measurements of channels which are not accessible *in situ* to patch-clamp studies. Comparative studies have shown that ion channels reconstituted with this procedure retain their channel properties (Criado & Keller, 1987; Keller et al., 1988; Berrier et al., 1989; Riquelme et al., 1990, 1993).

In this work, we have successfully applied the patch-clamp technique to UCP to study its channel properties. For this purpose, isolated UCP was reconstituted into proteolipsomes which were further fused into giant liposomes. Single-channel recordings on patches excised from the giant liposomes allowed us to demonstrate nucleotide-sensitive Cl^- channel activities of UCP. Further, we have investigated the influence on the channel properties of various potential factors for the sake of comparison with the Cl^- transport

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[1] Abbreviations: UCP, uncoupling protein; PC, phosphatidylcholine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; mCS, mitochondrial centum-picosiemens channel; Mes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylendiaminetetraacetic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; CFTR, cystic fibrosis transmembrane regulator.

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activity reported earlier. The anion channel exhibited by UCP under these conditions reflected Cl^- fluxes many order of magnitude higher than the carrier-type Cl^- transport activity reported previously.

EXPERIMENTAL PROCEDURES

Materials. Azolectin (type-IIS) was purchased from Sigma. Prior to use, it was washed with acetone and dried in vacuo. Phosphatidylcholine (PC) was isolated from hen egg yolk and purified over Al_2O_3 (Klingenberg et al., 1986). *n*-Decylpentaoxyethylene ($C_{10}E_5$) was obtained from Bachem AG (Basel). Other reagents were of analytical grade.

Isolation of UCP. UCP was isolated from a $C_{10}E_5$ solubilizate of brown adipose tissue mitochondria according to the procedure of Klingenberg and Winkler (1985). The detergent was removed by Biobeads, and a PC suspension was added to PC/UCP = 10 (wt/wt). The preparation had a single Coomassie blue band at 33 kDa on the Laemmli 12.5% polyacrylamide gel. Protein concentration was determined by the method of Lowry et al. (1951). The purity of the preparation was assessed from the [^{14}C]GTP binding capacity using the anion exchange method (Klingenberg et al., 1986). The binding capacities of 10−12 μmol of GTP/g of protein indicated a purity of 60−75% UCP in the preparation.

Reconstitution of UCP into Giant Liposomes. A freeze/thawing procedure was employed to prepare the proteoliposomes (Schwarz et al., 1994) as follows. A stock suspension of azolectin was prepared by sonicating acetone-washed azolectin (50 mg/mL) on a Branson sonifier for 10 min in a buffer consisting of 5 mM Mes, 5 mM Tris, pH 7.4. To 300 μL of this suspension was added 150 μg of UCP. After incubation for 20 min at 0 °C, the whole mixture was treated with Biobeads to remove any traces of residual detergent. The phospholipids containing the protein were recovered by centrifugation in an airfuge. This preparation (lipid/protein ≈ 100) was stored in liquid nitrogen until use. A stock azolectin suspension (50 mg/mL) was added to this preparation to a final lipid/UCP ratio of 1400. After sonication for 10 min at 0 °C, the mixture was diluted 10-fold in the standard buffer consisting of 100 mM KCl, 2 mM $MgCl_2$, 5 mM Mes, and 5 mM Tris, pH 7.4. Vesicles were obtained by quickly freezing in liquid nitrogen and slowly thawing at 0 °C. Giant liposomes were generated by a dehydration/rehydration treatment of these vesicles (Schmid et al., 1989).

Patch-Clamp Measurements. The standard method of Hamill et al. (1981) was used to perform single-channel recordings. Pipet microelectrodes for the patch-clamp measurements were fabricated from borosilicate glass capillaries (type GC150TF-10, Clark Electromedical Instruments). In the standard buffer, the pipet had a resistance of 20−50 MΩ.

To a 3.5 cm petri dish containing 2 mL of standard buffer was gently added 1 μL of the giant liposomes to the center of the dish bottom. After 15 min, the giant liposomes settled tightly on the bottom of the dish. The giant liposomes viewed with an inverted phase contrast microscope had a spherical appearance with a diameter of typically 5−100 μm and consisted of unilamellar, paucilamellar, and multilamellar liposomes. Unilamellar liposomes were selected for their appearance as faint rings according to Keller et al. (1988). Usually tight seals of 4−30 GΩ formed immediately. In some cases, a slight suction was applied to the pipet interior in order to obtain seals. The pipet was slowly withdrawn from the giant liposome, resulting in an excised patch ("inside-out" configuration). Recordings were performed on seals of >10 GΩ. Most of the patches were stable for a few minutes, while about 30% of them lasted 10−50 min. The channel properties did not change when recorded up to 50 min. The polarity given in the text refers to that of the pipet interior since the bath was grounded. A standard agar−KCl bridge was used in ion selectivity measurements to avoid any liquid junction potential. The orientation of UCP molecules in the excised membrane patch was determined by GTP inhibition experiments. The UCP has a right-side-out orientation (i.e., mitochondrial cytosol side facing the bath) when the channel activity was inhibited by GTP added to the bath. An inside-out-oriented UCP channel (mitochondrial matrix side facing the bath) can only be inhibited by GTP added in the pipet but not inhibited by GTP present in the bath medium.

Single-channel current data were recorded on a digital tape recorder (DTR-1200, Biologic) at a sampling rate of 40 kHz after filtering through an eight-pole low-pass bessel filter at 1 kHz for data presentation and at 2 kHz for data analysis. The data were transferred to an IBM 386 computer at a sampling rate of 33 kHz for analysis with the pClamp programs (Axon Instruments).

RESULTS

General Properties of the Channel Activities. Patch-clamp measurements were performed on membrane patches excised from unilamellar giant liposomes. In 36% of the patches (n = 197), we observed channel activities. Figure 1A shows typical single-channel currents recorded on a patch in symmetrical standard buffer (100 mM KCl, 2 mM $MgCl_2$, and 5 mM Mes/Tris, pH 7.4). At −100 mV, downward square current deflections were recorded which correspond to channel openings. The channel was occupied with slow open−closure transitions with durations of >10 ms superimposed by brief events or flickerings with a duration of ∼1−2 ms. Current transitions revealed two equal conductance states as defined by the current levels for the channel closed state (C), the first open state (O1), and the second open state (O2), with amplitudes of 9.7, 18.5, and 27.1 pA, respectively. At lower negative potentials (−60 mV and −30 mV), the channel was most of the time in the second open state (O2). At +30 mV, upward current transitions represent channel openings. The channel exhibited slow open−closure transitions, similar to those observed at low negative potentials (−60 and −30 mV). However, when the potential was more positive at +60 mV, fast flickerings appeared, where also the three current levels were clearly differentiated. At V_{hold} > +80 mV, openings at O2 became so rare that only one open state was seen. This voltage-dependent closure of one conductance state was confirmed in all recordings. The second uppermost trace shows a section of the recordings at +100 mV on an expanded time-scale. Figure 1B presents the data of current levels in the closed state (C) and the first (O1) and second (O2) open states. At V_{hold} between −80 mV and +40 mV, the channel is mostly in the open state. The unknown current level for the closed state was assigned (dashed lines) by extrapolation from known leak current levels (closed circles), which increases linearly with V_{hold}. The $I-V$ plot, shown in the inset to

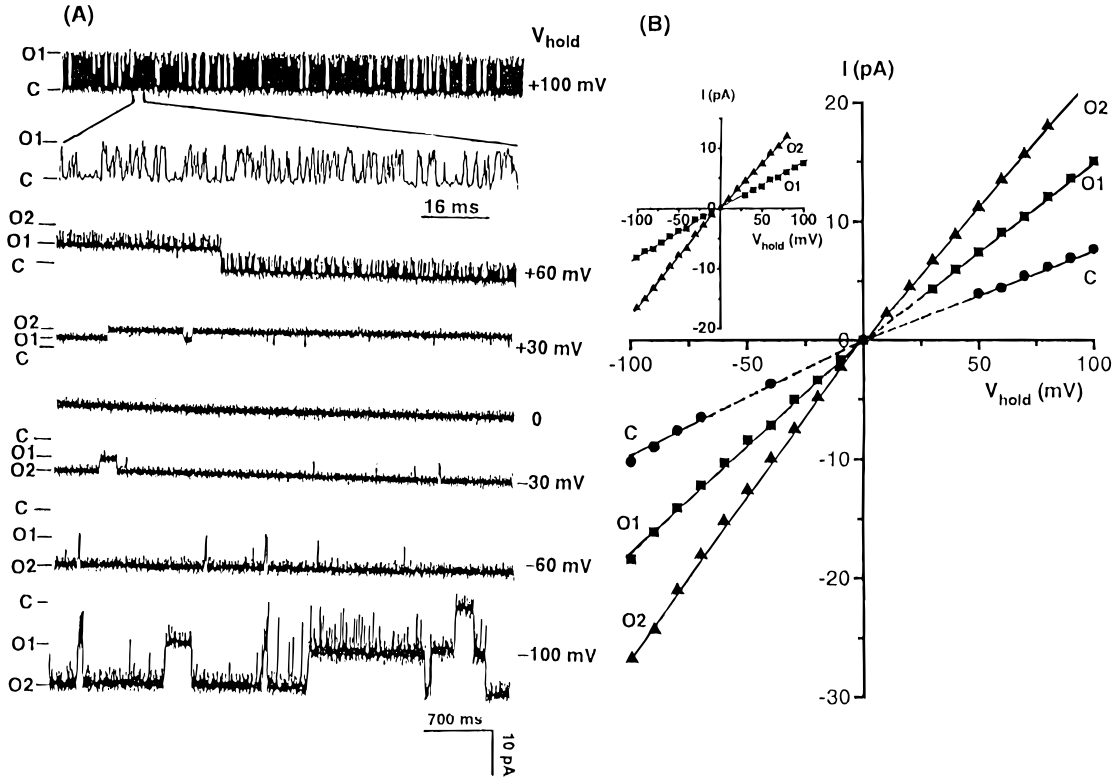

FIGURE 1: Single-channel currents and the current−voltage relationship of reconstituted UCP. (A) Single-channel currents recorded at various holding potentials (V_{hold}). C, O1, and O2 denote the three current levels when both channels are closed, one channel is open, and both channels are simultaneously open (see text). The second uppermost trace was a section of recordings at +100 mV expanded 44-fold in the time scale. (B) Current−voltage ($I-V$) relationship. Plotted are the electrical current levels of the closed (C), first open (O1), and second open (O2) states. Leak currents which are not directly observed in the records are extrapolated (dashed line) from the other known levels (solid line and circles). Both conductance states show a slight inward-rectification with a conductance of 75 pS at positive potentials and 84 pS at negative potentials. Recordings were performed on an excised patch (Experimental Procedures) in symmetrical standard buffer consisting of 100 mM KCl, 2 mM $MgCl_2$, 5 mM Mes, and 5 mM Tris, pH 7.4. The pipet contained additionally 1 mM $CaCl_2$.

Figure 1B with current data after correction for leak currents, reveals two open states with equal conductance; the channel exhibited a slight inward-rectification with a unit conductance of 75 ± 6 pS at positive potentials and 84 ± 8 pS ($n = 8$) at negative potentials. As will be shown later, the channel current is carried by Cl^- ions.

Patches excised from giant liposomes containing no UCP were electrically silent at V_{hold} between −160 and 160 mV ($n = 4$; not shown). This finding is consistent with previous reports (Criado & Keller, 1987; Keller et al., 1988; Schmid et al., 1988, 1989; Riquelme et al., 1990; Schwarz et al., 1994). Our standard procedure employs a lipid/protein ratio of 1400 (wt/wt) for the reconstitution. We also increased the protein content to a lipid/protein ratio of 700. Recordings on these liposomes revealed up to six channel openings in the patch. However, the patches were less stable. At lipid/protein = 2800, channel activities were observed in only about 20% of the patches ($n = 24$). Despite the lower channel density, the same current fluctuations characteristic of the two equal conductance states as shown in Figure 1A were observed in all five patches.

In line with the previously published procedure for generating giant liposomes for patch-clamp measurements (Schwarz et al., 1994), we used a buffer containing 2 mM Mg^{2+} and in the pipet additionally 1 mM Ca^{2+}. We investigated whether these ions can modulate the channel properties. With giant liposomes prepared in the absence of Mg^{2+} and Ca^{2+}, the same channel conductance and open probability (P_{open}) was observed when first Mg^{2+}, Ca^{2+}, and then EGTA were added to the bath. However, the excised patches were less stable in the absence of these ions. Therefore, Ca^{2+} (in pipet) and Mg^{2+} were included in our standard measurements.

Inhibition by Purine Nucleotides. Nucleotide binding has served from the beginning as a tool for identifying the UCP (Heaton et al., 1978; Lin & Klingenberg, 1980). Purine nucleotides (GTP, GDP, ATP, and ADP) bind with a K_D of 3−12 μM (pH 7.4) to UCP. It was established previously that nucleotides can inhibit the UCP-catalyzed H^+ transport (Nicholls, 1979; Klingenberg & Winkler, 1985; Winkler & Klingenberg, 1992) as well as Cl^- transport (Nicholls & Lindberg, 1973; Jezek et al., 1990). Therefore, nucleotide inhibition should serve as a criterion for associating the chloride channel activity with UCP.

GTP can completely inhibit the chloride channel. As shown in Figure 2A, at +80 mV only one open state was active, exhibiting fast flickerings in the absence of GTP (trace a). When 50 μM GTP was added to the bath medium, the currents flickered more toward the closed state (trace b). At 500 μM GTP, the inhibition was nearly complete as shown by the very rare flickerings (trace c), and at 800 μM GTP, the channel was completely inhibited (trace d). When GTP was removed by perfusion with the standard medium, the channel activities were fully restored (trace e), indicating that the inhibition by GTP is reversible.

To further evaluate the nucleotide inhibition, we estimated open probabilities (P_{open}) from amplitude histograms (not shown). The P_{open} in the absence of GTP was 0.63 (referred

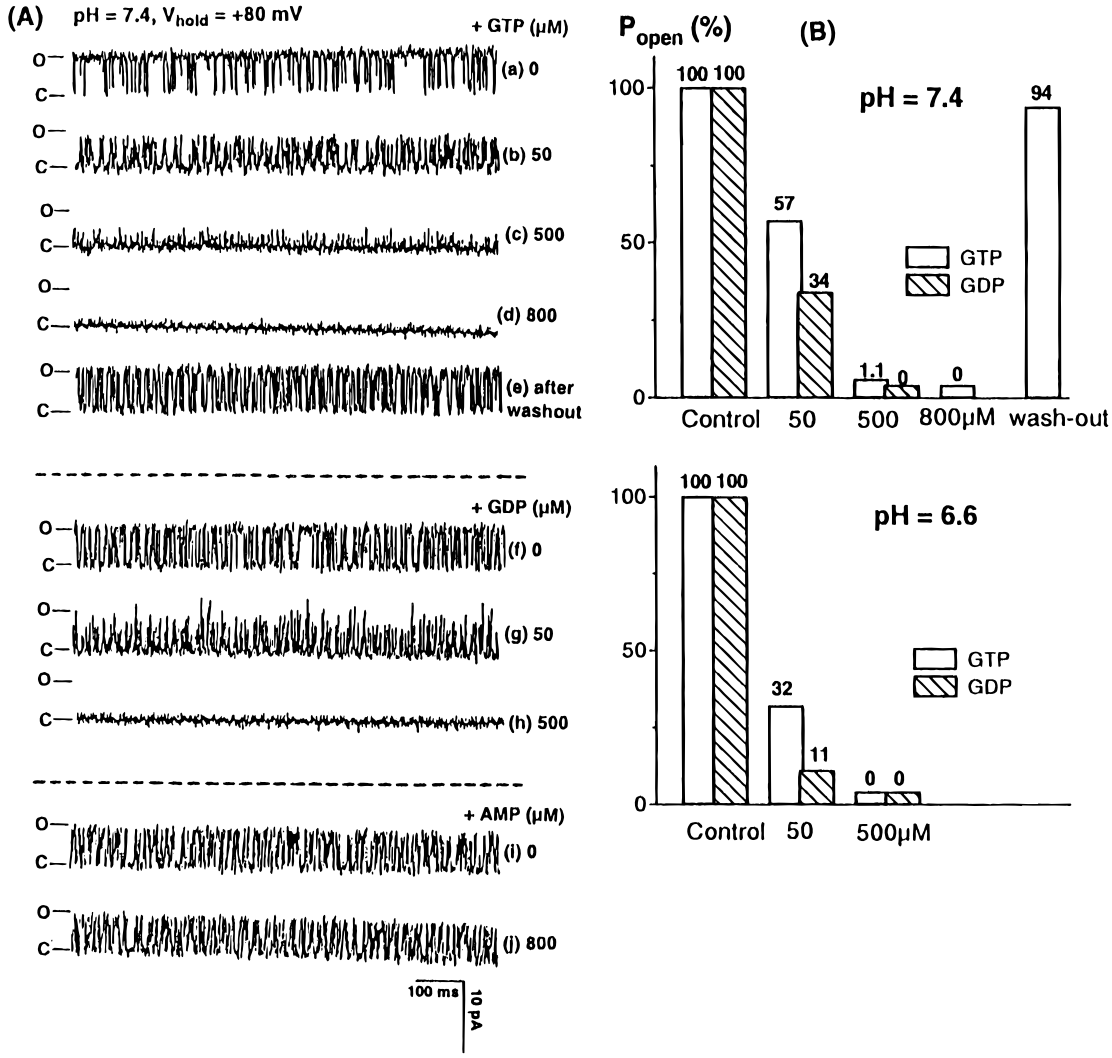

FIGURE 2: Inhibition of the Cl^- channel of reconstituted UCP by nucleotides. (A) Single-channel recordings were obtained in standard buffer at pH 7.4 and +80 mV in the presence of GTP (traces a−c), GDP (traces d−f), and AMP (traces i and j). (B) Open probability (P_{open}) in response to inhibition by GTP (open bars) and GDP (hatched bars) at pH 7.4 and 6.6. P_{open} values were estimated from the respective histograms (not shown). Each experiment was repeated 5 times with the same results.

to as 100%, Figure 2B). In the presence of 50 and 500 μM GTP, it decreased by 43 and 99%, respectively. At 800 μM, the P_{open} was 0, whereas removal of the GTP restored the P_{open} to 94%. GDP had similar inhibitory effects, but inhibited even more strongly than GTP. GDP produced a decrease in P_{open} from 0.56 to 0.19 or by 66% at 50 μM (trace g) and completely inhibited the channel at 500 μM (trace h). ATP and ADP caused a similar inhibition (figure not shown). AMP, which does not inhibit H^+ transport (Huang & Klingenberg, 1996), had negligible influence on the channel activity even present at a high concentration (800 μM, traces i and j).

The above experiments were performed at pH 7.4. Since the affinity of nucleotide binding to UCP was stronger at lower pH (Klingenberg, 1988), we also carried out the measurements at pH 6.6. The inhibition was indeed stronger than at pH 7.4. As shown in Figure 2B, the P_{open} decreased by 68% (versus 43% at pH 7.4) in the presence of 50 μM GTP, and by 89% (versus 66%) in the presence of 50 μM GDP. At a concentration of 500 μM, both GTP and GDP completely inhibited the channel.

Orientation of UCP. The current fluctuations change from slow open−closure transitions to fast flickerings as the holding potential increased from −100 mV to +100 mV, displaying a "polarity" in the voltage-dependent current fluctuations (Figure 1A). However, recordings on about 30% of the patches showed a reversed polarity; i.e., slow transitions occur at positive potentials whereas fast flickerings appear at $V_{hold} < -60$ mV. We observed further that nucleotide added to the bath was inhibitory only when the flickerings occurred at $V_{hold} > +60$ mV (see Figure 2). As shown in Figure 3A, the channel in this patch (trace a) exhibited the same polarity as the one shown in Figure 1 and Figure 2, judging from the slow transitions at −80 mV. Here we applied a negative potential in order to demonstrate nucleotide inhibition also at negative V_{hold}. The level for the closed state (C) was assigned from a comparison of the leak currents as described in Figure 1B; 50 μM GTP caused a flickering of channel openings (trace b). In the presence of 800 μM GTP, the channel was completely inhibited (trace c). The channel from another patch (Figure 3B) had an opposite polarity compared to Figure 3A, judging from the fast flickerings when recorded at −80 mV. Up to 800 μM GTP exhibited little inhibition since the P_{open} decreased only about 9% (traces d and e). The same applies to the inhibition at +80 mV (Figure 3C). The small transitions (trace f, Figure 3C) are probably occasional substates, which were not inhibited by GTP ($n = 4$). When GTP was added to

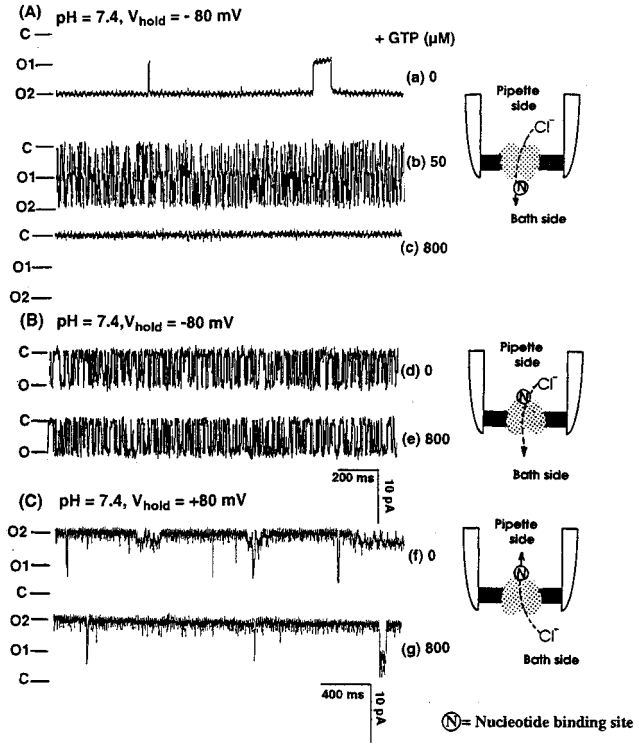

FIGURE 3: Orientation of UCP in the patch membrane. (A) GTP added to the bath medium inhibits the Cl^- channel which displays slow open−closure transitions at negative potentials (cf. Figure 1). (B, C) GTP does not inhibit a channel which has an opposite gating polarity as shown by the current fluctuations. The schemes illustrate a UCP channel with two orientations in the patch membrane. With a right-side-out orientation (A), the nucleotide binding site is facing the bath side, whereas with an inside-out orientation (B, C), GTP added to the bath is not accessible to the nucleotide binding site. The arrows of the dashed lines represent the direction of Cl^- flux. Results were confirmed in four separate patches.

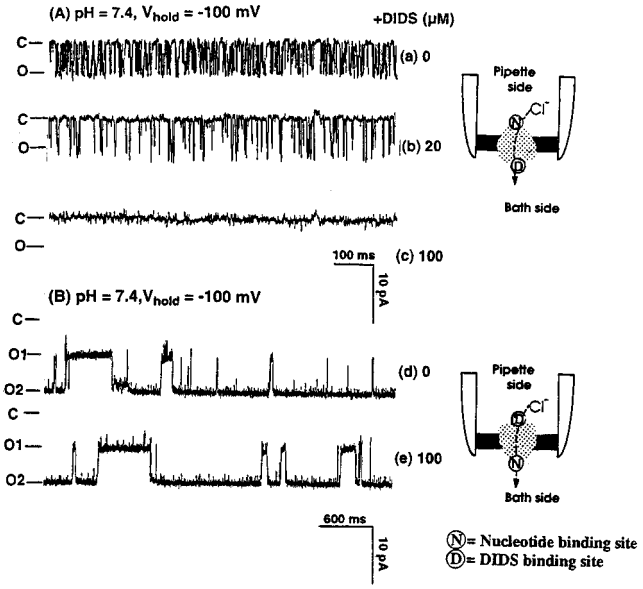

FIGURE 4: Blocking of the Cl^- channel by DIDS. (A) DIDS added to the bath medium blocks an inside-out channel; (B) DIDS does not block a right-side-out-oriented UCP channel. Measurements were performed in symmetrical standard buffer at −100 mV and pH 7.4 ($n = 3$).

both the pipet and bath, namely, on both sides of the membrane, we observed inhibition in all cases. These findings can be interpreted in terms of UCP orientation in the excised patch: a UCP channel displaying fast flickerings at $V_{hold} > +60$ mV has a right-side-out orientation (i.e., UCP cytosol side facing the bath), whereas a channel exhibiting a reversed polarity is oriented inside-out (matrix side facing the bath).

pH Dependence and Effects of Fatty Acids. Several studies have shown that the Cl^- transport activity measured in brown adipose tissue mitochondria (Nicholls &d Lindberg, 1973) and in proteoliposomes (Jezek et al., 1990) displays a rather weak pH dependence. Single-channel recordings were performed in symmetrical 100 mM KCl where the pH of the bath medium was varied while the pH of the pipet medium was kept at 7.4. An analysis of the recordings revealed little changes at pH 6.6, 7.4, and 8.3 ($n = 4$), suggesting that the Cl^- channel exhibit weak if any pH dependence (not shown).

Long-chain (C ≥ 10) fatty acids are known to activate the H^+ transport of UCP (Nicholls & Locke, 1984; Winkler & Klingenberg, 1994). Fatty acids had no effects on the Cl^- transport activity as shown in swelling experiments by Nicholls and Lindberg (1973), whereas an inhibition by fatty acid was claimed by Jezek and Garlid (1990) in their work on proteoliposomes. However, here lauric acid had no observable effects on the channel activity (traces not shown), even present at 200 μM known to fully activate the H^+ transport activity in reconstituted systems (Winkler & Klingenberg, 1992, 1994). Hexanesulfonic acid, a fatty acid analog previously reported by Jezek and Garlid (1990) to inhibit Cl^- transport in the proteoliposomes, did not alter the Cl^- channel properties at concentrations up to 1.5 mM.

Effects of Mersalyl and DIDS. Mercurial reagents were reported to react with some critical cysteine group(s) and switch some mitochondrial exchange-type carriers into the unidirectional transport mode (Dierks et al., 1990, 1994). Even when mersalyl (1 mM) was applied on both sides of the membrane, no effect on the channel activity was seen. Although there is so far no report on 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) inhibition of Cl^- transport in intact mitochondria or in a reconstituted system, we tested whether the Cl^- channel blocker (Franciolini & Adams, 1994) can block the UCP-mediated Cl^- current. As shown in Figure 4, the fast flickerings recorded at −100 mV indicate an inside-out-oriented UCP channel in this patch (trace a). A strong blockage was observed with 20 μM DIDS in the bath (trace b), and with 100 μM DIDS, this channel was completely blocked (trace c). Extensive perfusion of the bath with the buffer did not reactivate the channel, suggesting that DIDS blocks the channel irreversibly. Interestingly, at even up to 1 mM DIDS had no effects on a right-side-out UCP channel (traces d and e). These results suggest that DIDS can bind covalently at the matrix side of UCP (i.e., opposite to the GTP binding site) and block the UCP channel.

Ion Selectivity. The anion selectivity was investigated by measuring the shift in the $I-V$ plots on substituting the bath Cl^- with a different anion. Reversal potentials (V_{rev}) were estimated, and anion permeabilities relative to Cl^- were calculated from the Goldmann−Katz−Hodgkin equation (Hille, 1984). As illustrated in Figure 5A, on substituting Cl^- with F^-, current amplitudes of the anion channel deviated progressively from the control value in 100 mM KCl as the V_{hold} increased from −100 mV to +100 mV. At $V_{hold} = -100$ mV, the channel current was nearly constant (9.0 pA

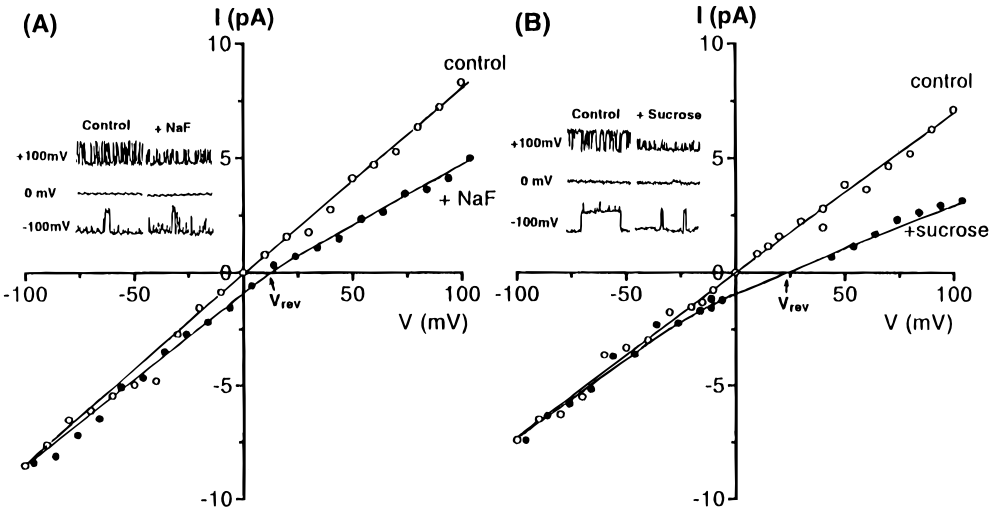

FIGURE 5: Ion selectivity of the Cl^- channel of reconstituted UCP. Single-channel recordings were obtained at pH 7.4. (A) $I-V$ plots and traces showing the effects of substitution of 100 mM Cl^- with 100 mM F^- in the bath. (B) $I-V$ plots and traces showing the effects of substitution of 100 mM KCl with 28 mM KCl plus 144 mM sucrose. Reversal potentials (V_{rev}) were estimated from the shift in the $I-V$ plots. A salt bridge was used during the measurements to avoid liquid junction potential.

Table 1: Ion Selectivity of the Chloride Channel of Reconstituted UCP[a]

test ion	V_{rev} (mV)	P_{rel}	test ion	V_{rev} (mV)	P_{rel}
Cl^-	0	1.00	NO_3^-	25.0	0.37
Br^-	3.2	0.90	SO_4^{2-}	13.5[b]	0.26
F^-	13.2	0.59	HPO_4^{2-}	16.5[c]	0.18
SCN^-	16.2	0.53	gluconate	24.8[d]	0.12
I^-	20.0	0.45	K^+	25.0[e]	0.06

[a] Ion permeabilities (P_{rel}) relative to the Cl^- pemeability were determined from the reversal potential (V_{rev}) in the $I-V$ plots of single-channel currents measured in the standard medium (pH 7.4) before and after substitution of the bath Cl^- with the test ions. [b] Bath medium contained 67 mM K_2SO_4. [c] Bath medium contained 67 mM K_2HPO_4. [d] Bath medium contained 28 mM KCl plus 72 mM potassium gluconate. [e] Bath medium contained 28 mM KCl plus 144 mM sucrose.

versus the control 8.6 pA), whereas at +100 mV it decreased from 7.6 pA to 4.5 pA (see traces). From the $I-V$ plot, a reversal potential of +13.2 mV was obtained, which corresponds to a permeability ratio of $P(F^-)/P(Cl^-) = 0.59$. In the same manner, we have measured the relative permeabilities (P_{rel}) for several anions (Table 1). Of the various monovalent anions tested, only Br^- displayed a permeability comparable to that of Cl^- [$P(Br^-)/P(Cl^-) = 0.90$]. F^- and I^- were nearly half as permeable as Cl^-; furthermore, their P_{open} at +100 mV decreased by 19% and 5%, respectively. SCN^- and NO_3^- also displayed lower permeabilities. For the large anion gluconate, a V_{rev} of +24.8 mV was measured on replacing 100 mM KCl with 28 mM KCl plus 72 mM potassium gluconate, which corresponds to a permeability 8-fold lower than that for Cl^-. Thus, the permeability can be arranged in the order $Cl^- > Br^- > F^- > SCN^- > I^- > NO_3^- >>$ gluconate. When the bath medium was replaced with 67 mM K_2SO_4 and K_2HPO_4, V_{rev} values of +13.5 mV for sulfate and +16.5 mV for phosphate were determined, suggesting that both ions are permeant. From the constant field theory adapted by Lewis (1979) for measuring the permeability of divalent ions, we estimated relative permeabilities of 0.26 and 0.18 for SO_4^{2-} and HPO_4^{2-}, respectively.

The permeability of K^+ was estimated in asymmetric KCl solutions. As shown in Figure 5B, a shift of +25.0 mV in the $I-V$ plots was obtained on replacing the bath medium (100 mM KCl) with 28 mM KCl plus 144 mM sucrose. This

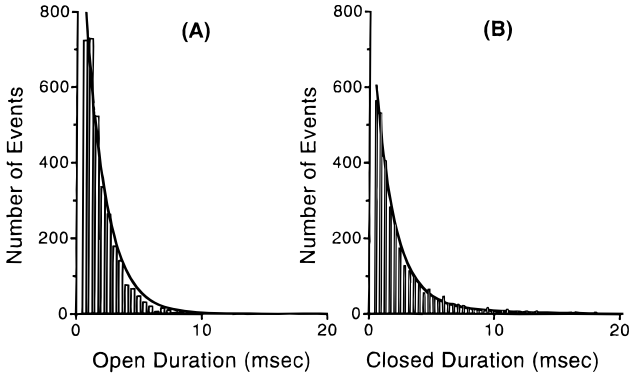

FIGURE 6: Distribution of the open and closed times of the fast flickerings of the right-side-out-oriented UCP channel. Histograms were constructed from single-channel recordings obtained in standard buffer at +100 mV. (A) Open time distribution was best fitted with a single-exponential function yielding a mean open time of 1.7 ms. (B) Closed time distribution was best fitted with two exponentials with mean closed times of 1.6 and 5.4 ms.

value is lower than the expected value of +29.0 mV if only Cl^- would travel through the channel. This discrepancy is accounted for by assuming a small K^+ permeability [$P(K^+)/P(Cl^-) \approx 0.06$]. Substitution of K^+ with Na^+ or large impermeant cations (tetraethylammonium chloride or triethanolamine hydrochloride) did not alter the channel properties. These results show that Cl^- is the sole transported ion.

Kinetics of Fast Flickerings, Open Probability, and Concentration Dependence. The right-side-out-oriented UCP channel displays slow open−closure transitions at lower holding potentials ($V_{hold} < +60$ mV), which makes a statistical analysis of the kinetics difficult. At high positive V_{hold}, the channel shows fast flickerings, allowing an analysis of the open and closed time distributions. The open time histogram (Figure 6A) of single-channel currents recorded at +100 mV was best fitted with a single-exponential equation with a mean open time of 1.7 ms, whereas the closed time histogram (Figure 6B) was best fitted with two-exponential terms with mean closed times of 1.6 and 5.4 ms.

The open probability (P_{open}) decreases only slightly as the V_{hold} decreases in the negative potential range (Figure 7A). However, a steeper decrease is observed in the positive potential range. From a fitting of the voltage dependence

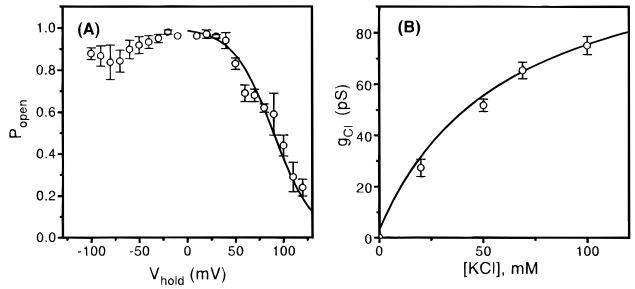

FIGURE 7: Open probability (P_{open}) and concentration dependence of the right-side-out-oriented UCP channel. Single-channel recordings were obtained in the standard buffer at pH 7.4. (A) Open probability (P_{open}) was estimated from the respective recordings on four separate patches. The dependence between 0 and +120 mV was fitted (solid line) according to the Boltzmann distribution $P_{open} = \{1 + \exp[zF(V - V_o)/RT]\}^{-1}$ (Behrends et al., 1989), where z is the gating charge, V the applied voltage, V_o the V_{hold} at which the channel is half open, R the gas constant, and T the absolute temperature. From the fitting, a gating charge (z) of 1.2 ± 0.1 and a V_o of 89.7 ± 1.7 mV ($n = 4$) were obtained. (B) Concentration dependence of the Cl^- channel. The pipet contained standard KCl (100 mM) medium at pH 7.4, while the bath Cl^- concentration was replaced with gluconate. A fit (solid line) according to the equation $g = g_{max}[Cl^-]/([Cl^-] + K_m)$ assuming a $g_{max} = 120$ pS yields a $K_m = 63 \pm 3$ mM ($n = 3$).

in this range according to the Boltzmann distribution (Behrends et al., 1989), we estimated an effective gating charge of 1.2 ± 0.1, which suggests that 1.2 charges are moved to open or close the channel. The potential at which the channel was half open (V_o) was 89.7 ± 1.7 mV. At $V_{hold} = 0$ mV, we infer from the voltage dependence that the channel is mostly in the open state.

To investigate the concentration dependence, single-channel conductances were measured on the same patch at different bath Cl^- concentrations while keeping the pipet concentration constant (100 mM KCl). The results obtained at +100 mV are presented in Figure 7B. The Cl^- conductance showed a saturation as the concentration increased. The measured conductance (g) approached a maximum (g_{max}) at 120 pS as estimated from the double-reciprocal plot (not shown). A theoretical fitting with the equation $g = g_{max}[Cl^-]/([Cl^-] + K_m)$ assuming $g_{max} = 120$ pS yields a $K_m = 63 \pm 3$ mM, which corresponds to the Cl^- concentration at which the channel exhibits its half-maximum conductance.

DISCUSSION

By transporting small ions such as H^+ and Cl^-, UCP seems to be functionally the simplest member of the mitochondrial carrier family (Aquila et al., 1987; Runswick et al., 1990). It is therefore of great interest to examine whether UCP also can exhibit channel properties. Studies on mitoplasts indicated the presence of various channels in the inner mitochondrial membrane [reviewed in Tedeschi and Kinnally (1994)]. Very recently the isolated and reconstituted ADP/ATP carrier was shown to exhibit channel behavior with high conductance and low specificity (Brustovetsky & Klingenberg, 1996). Also, ionic channel properties were observed in reconstituted chloroplast phosphate/triosephosphate carrier (Schwarz et al., 1994). In less related cases, transporters such as Ca^{2+}-ATPase (Shamoo & MacLennan, 1975) and Na^+,K^+-ATPase (Last et al., 1983; Mironova et al., 1986) were shown to behave like ion channels.

General Chloride Channel Properties of UCP. Patch-clamp meaurements on membranes with reconstituted UCP in 100 mM KCl reveal stable Cl^- currents with a unit conductance of ~75 pS. At $V_{hold} > +60$ mV, channel gating switches from slow open−closure transitions to fast flickerings, indicating an asymmetric ion translocation path within the channel, similar to the chloroplast phosphate/triosephosphate carrier (Schwarz et al., 1994). The channel discriminates largely against cations with $P(Cl^-)/P(K^+) \approx 17$. A small cation permeability associated with anion translocation is a characteristic of many Cl^- channels (Franciolini & Nonner, 1987; Petris et al., 1994). The permeability ratio $P(Cl^-)/P(K^+)$ for the UCP channel is similar to that (11) observed for the CFTR Cl^- channel (Rich et al., 1991). We measured a halide selectivity order of $Cl^- > Br^- > F^- > I^-$, which differs from that of the large group of background Cl^- channels (Petris et al., 1994), but is close to that observed for the CFTR channel (Anderson et al., 1991). According to Eisenman's theory of ionic selectivity (Eisenman & Horn, 1983), this sequence indicates that the channel has a high-affinity site for the anion. The open probability decreases as the V_{hold} increases with an effective gating charge of 1.2.

Although the protein preparation was about 70% pure, we conclude that this channel is an integral property of UCP for the following reasons. First, the channel can be inhibited reversibly by purine tri- and diphosphates, but not by monophosphate. This feature is consistent both with the nucleotide specificity of UCP (Lin & Klingenberg, 1982; Klingenberg, 1988) and with the nucleotide inhibition of Cl^- transport (Nicholls & Lindberg, 1973; Jezek et al., 1990) as well as of H^+ transport (Winkler & Klingenberg, 1985; Huang & Klingenberg, 1996). The concentrations of GTP and GDP required to inhibit half of the channel openings were approximately 50 μM, which correspond to free concentrations of 3 μM for GTP and 9 μM for GDP when the complexing effect by 2 mM Mg^{2+} is corrected for (Smith & Alberty, 1956). These data agree closely with the K_D values at pH 7.4 of 5 μM for GTP (Klingenberg, 1988) and 10 μM for GDP (Lin & Klingenberg, 1982). The observed stronger inhibition at pH 6.6 than at pH 7.4 is expected from the pH dependence of nucleotide binding (Klingenberg, 1988; Huang & Klingenberg, 1995). Second, the channel properties described in this work differ from those of any known mitochondrial anion channels (see below).

An intriguing phenomenon is the consistent occurrence of two equal conductance states. Even when we "diluted" the protein in the giant liposomes on purpose, recordings still revealed the same two open states (cf. Figure 1A) albeit at a cost of much lower probability of finding the channels. The fact that the two conductance states are either both completely inhibited by GTP (for UCP oriented right-side-out in the membrane patch, $n = 14$) or not inhibited (for UCP oriented inside-out, $n = 8$) further argues for an interdependency either by a two-stage opening of a monomer channel or by a dimer channel, since a random orientation of two separate channels in the membrane would have resulted in half-inhibition by excess GTP, which was not observed throughout this work. Thus, either the channel has a total conductance of 150 pS, or within the dimer each UCP monomer possesses a Cl^- channel with a conductance of 75 pS.

Comparison with the Cl^- Transport Activity of UCP. Both the Cl^- channel described in this work and the Cl^- transport activity of UCP reported earlier can be inhibited by purine nucleotides. Both pathways exhibit weak pH dependence

and no fatty acid activation. However, the unit conductance of the UCP Cl^- channel (75 pS) is approximately 6 orders of magnitude higher than that measured for the Cl^- transport activity. Also these pathways exhibit different anion selectivity (Nicholls & Lindberg, 1973; Jezek et al., 1990). We assume that both activities use the same Cl^- translocation path within the UCP, but they represent two conducting states. However, what factors determine the conducting state remains a challenge of our future studies.

A similar discrepancy was observed with the phosphate/triosephosphate carrier by Schwarz et al. (1994). Although the measurements in chloroplasts do demonstrate an approximately 16-fold increase in Cl^- transport activity when the Cl^- concentration was raised from 20 to 100 mM, where the transport rate reached approximately 40 s^{-1}, this value is still 6 orders of magnitude lower than that measured under patch-clamp conditions. These authors interpreted the high ion transport rate under patch-clamp conditions as a result of a carrier-to-channel transition induced at saturating ion concentrations. However, such a dramatic concentration-dependent increase was not shown in their patch-clamp measurements. Our results show that the UCP channel does not exhibit such an abnormal concentration dependence (Figure 7B).

Comparison with Other Mitochondrial Anion Channels. A comparison with known mitochondrial anion channels would further help eliminate the possibility that the Cl^- channel described here represents a contamination from other channels in brown adipose tissue mitochondrion. The ADP/ATP carrier (AAC) was shown recently to form a channel under similar conditions as used here for UCP (Brustovetsky & Klingenberg, 1996). However, this channel is dependent on a high Ca^{2+} concentration and has a high conductance (up to 500 pS in 100 mM KCl). It was blocked by bongkrekate, a specific inhibitor of AAC. The behavior of the AAC channel resembles those of the multiple conductance channel or megachannel reported earlier in mitochondria (Kinnally et al., 1991; Szabo & Zoratti, 1991, 1992). The "alkaline-induced anion-selective channel" opens at alkaline pH with a conductance of ~40 pS in 100 mM KCl (Antonenko et al., 1994), similar to the inner mitochondrial anion channel (IMAC; Garlid & Beavis, 1986; Beavis, 1989). The "mitochondrial centum-picosiemens" channel (mCS) has a conductance of 107 pS in 150 mM KCl (~94 pS in 100 mM KCl). Although this channel has a conductance close to that of the Cl^- channel reported here, we argue that it is not identical to the UCP channel for the following reasons. First, nucleotide inhibition has not been confirmed with the mCS. Klitsch and Siemen (1991) demonstrated an inhibition by nucleotides under "whole-mitoplast" conditions with brown adipose tissue mitochondria, but in our opinion, the nucleotide could interact with various other components in the mitoplast so that the observed effect could not be exclusively assigned on the mCS. Also the inhibition did not agree with the properties expected of nucleotide binding to UCP. For this reason, they concluded that the mCS channel was distinct from UCP. Second, the mCS channel displays a strong rectification so that it opens at matrix positive potentials but closes at negative potentials. In contrast, the UCP channel tends to close at matrix positive potentials. Further, the effective gating charge of ~3 (Ballarin et al., 1994) differs largely from that for the UCP channel (1.2); the same applies to the V_o (4−13 mV versus 90 mV for the UCP channel). Third, the Ca^{2+} sensitivity (Kinnally et al., 1991) and lack of DIDS blockage (Sorgato et al., 1989) distinguish the mCS channel from the UCP channel. Finally, the channel gating kinetics also differ (Tedeschi & Kinnally, 1994; Klitsch & Siemen, 1991; Sorgato et al., 1989). On the outer mitochondrial membrane, the voltage-dependent anion channel (VDAC) or mitochondrial porin has a high conductance of ~500 pS in 100 mM KCl and selects poorly between cations and anions [$P(Cl^-)/P(K^+) \approx 1.8$; Colombini, 1989]. Characteristically, it is strongly voltage-dependent so that closures occur already at >40 mV.

In conclusion, the Cl^- channel described in this work shares no common features with other mitochondrial channels in terms of single-channel conductance, pH dependence, and modulations by Ca^{2+} or Mg^{2+}. Rather, this 75/150 pS Cl^- channel is an integral property of the UCP itself.

Role in Regulation. Whether the Cl^- channel as well as the slow Cl^- transport has any physiological significance remains unresolved at this point. It would have to be directed only to the brown adipose tissue. However, here the main function of UCP is generally understood to be confined to transfer of H^+ which generates neutralization energy and thus heat. One may speculate that the UCP chloride channel plays a role in volume regulation in mitochondria as observed in swelling or shrinkages associated with cold- and warm-acclimation (Desautels & Himms-Hagen, 1980).

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