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Inner Mitochondrial Membrane Anion Channel is Present in Brown Adipocytes but is not Identical with the Uncoupling Protein

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Summary. Vesicles of inner mitochondrial membrane, mitoplasts, from rat brown adipose tissue were prepared by osmotic swelling and studied using the patch-clamp technique. Current events of a 107.8 ± 8.7 pS (n = 16, 21°C) channel were recorded in the mitoplast-attached mode. This channel was selective for anions and its kinetics resembled those of channels previously found in liver and heart mitochondria of mouse and ox. In whole-mitoplast mode each of five purine nucleotides (20 μM) blocked the channel. This is the first demonstration of pharmacological blockade of this type of channel. Although a similar anion channel in mouse and ox mitochondria was suggested to be the uncoupling protein (UCP) associated with nonshivering thermogenesis, we present several arguments against this possibility. Thus we describe a high-conductance, purine-nucleotide-binding, anion selective mitochondrial channel, that is not the UCP.

Key Words inner mitochondrial membrane · anion channel · thermogenin · uncoupling protein · purine nucleotides · guanosine 5′-diphosphate · brown adipose tissue

Introduction

Nonshivering thermogenesis in brown adipocytes is due to the unique ability of their mitochondria to uncouple the respiratory chain from ATP synthesis (for review *see* Nicholls, Cunningham & Rial, 1986). Uncoupling occurs via a 32-kDa protein in the inner mitochondrial membrane which works as a functional proton shunt. This protein is called thermogenin or uncoupling protein (UCP). It can be blocked by purine di- and trinucleotides (adenosine triphosphate, ATP; adenosine diphosphate, ADP; guanosine 5′-triphosphate, GTP; guanosine 5′-diphosphate, GDP). However, the block may be overridden by free fatty acids. In earlier experiments it was not clear whether the UCP limited ATP synthesis by proton transport—intriguing to think for short-circuiting the ATP-synthase—or if it transported anions in the opposite direction. Nicholls (1974; *see also* Nicholls & Lindberg, 1973; Nicholls et al., 1986) found a Cl^- permeability which seemed to be related to the UCP as the Cl^- ions competed with protons when passing the inner mitochondrial membrane. In addition the Cl^- pathway was blockable by purine nucleotides. However, fatty acids did not increase the Cl^- conductance as was the case with the proton pathway (Nicholls, Snelling & Rial, 1984).

Since the chemiosmotic theory of Mitchell (1966), it was believed that the inner mitochondrial membrane would be widely impermeable to ions other than those carried by the well-known transport proteins. However, Weiner (1975) found an additional halide anion permeability under some conditions. In 1987 Sorgato, Keller and Stühmer showed a Cl^- conductance in the inner mitochondrial membrane of liver cells. Using the patch-clamp method they found an anion channel with a single channel conductance of about 107 pS. They tested 12 common blockers of different species of ionic channels without success (Sorgato et al., 1989). Selwyn (1987) discussed a possible relation of this channel to UCP and nonshivering thermogenesis (NST) of brown adipose tissue. Here we show that a Cl^- channel with properties similar to the channel described by Sorgato et al. (1987, 1989) is present in the inner mitochondrial membrane of rat brown adipocytes. It can be blocked by purine di- and trinucleotides and surprisingly by guanosine 5′-monophosphate as well. From this and other arguments we conclude that the purine nucleotide-blockable anion conductance in the inner mitochondrial membrane of brown adipocytes and liver cells is distinct from the UCP.

Materials and Methods

Mitochondria were prepared from interscapular brown adipose tissue of 6–8 week old male Sprague-Dawley rats according to the method described by Cannon and Nedergaard (1987). Animals were reared at room temperature (21°C). The slow centrifugation

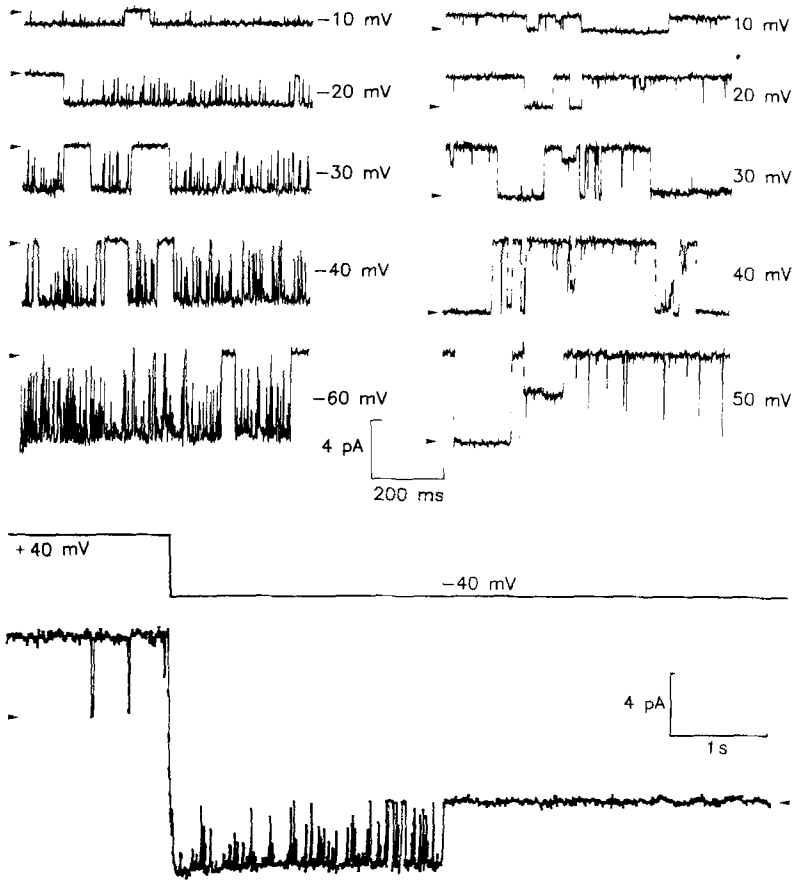

Fig. 1. Current recordings of anion channels in the inner mitochondrial membrane of brown adipocytes. Mitoplast attached. *Upper part:* Single channel current recordings at the indicated holding potentials. In order to remove deactivation of channels, the membrane was kept at +40 mV prior to records at negative potentials. Arrowheads mark levels at which channels are closed. Notice the prolonged open times at depolarizing potentials and occasional events with smaller amplitude. *Lower part:* Record at slower time scale demonstrating voltage and time-dependent deactivation of the anion channel. At +40 mV the channel mainly stayed in the open state (closed state marked by arrowheads). After changing to −40 mV, the channel started closing at an increasing rate for about 2½ sec and then stayed closed. The differences in the current amplitudes between arrowheads were due to leakage current

was done at 800 × *g* and the fast centrifugation at 9200 × *g* (10 min spin duration). The solution used for isolation contained (in mM): 250 sucrose, 5 K-HEPES, 1 EGTA. The mitochondria were kept on ice (0–4°C) for up to 36 hr in a solution containing (in mM): 150 KCl, 20 K-HEPES. Both solutions contained 0.1% bovine serum albumin and had a pH of 7.2. Bacterial contamination was excluded by assays performed by Dr. S. Bhakdi (Institut für Medizinische Mikrobiologie, Giessen).

Experiments were done in 35 mm Petri dishes at room temperature (21°C). The outer mitochondrial membrane was removed by hyposmotic treatment. About 2 μl of the mitochondria suspension was diluted in 2 ml of a solution containing 5 mM K-HEPES at pH 7.2. After 5–10 min 0.5 ml of a solution with five-fold the normal ionic strength (750 KCl and 100 K-HEPES at pH 7.2) was added to the bath to restore isotonic conditions. With 400 × phase contrast small fragile vesicles (3–5 μm diameter) could be observed, each with a dark spot (cap) at one side as shown in Fig. 1*D* of Sorgato et al. (1987). 5–15 GΩ seals were formed by approaching these mitoplasts with the pipette from the side opposite to the cap. Pipette solution contained (in mM): 150 KCl, 20 K-HEPES at pH 7.2. Bath solution was changed by means of a pump-driven "sewer pipe" system (Yellen, 1982). In the vicinity of the cap the presence of outer membrane fragments caused an increased probability of getting patches with the large voltage-dependent anion channel (VDAC).

Patch clamp followed the method of Hamill et al. (1981). Pipettes were polished down to give a resistance of 15–35 MΩ. Gentle suction was necessary only to move the free floating mitoplasts to the tip of the pipette. GΩ seals then formed spontaneously. In two typical experiments in the "whole-mitoplast mode" (20 and 23 MΩ pipette resistance) we found a series resistance of 384 and 350 MΩ and a membrane capacitance of 0.66 and 0.57 pF, respectively (for calculation *see* Schmidt, 1986). According to Sigworth (1983) a value of 0.5 pF would indicate a mitoplast diameter of 4 μm. Filter frequency was 2 kHz for histograms and 1 kHz for figures. The sample rate was set to 150 μsec. Signs of the potentials refer to the inner side of the membrane and inward currents always deflect downward. Further details of the recording technique were previously described (Weber & Siemen, 1989; Siemen & Weber, 1989).

Results

Single Channel Conductance and Kinetics

Square shaped current events with a mean single channel conductance (γ) of 107.8 ± 8.7 pS (n = 16, 21°C) were recorded when the patch pipette formed a GΩ seal with the mitoplast surface, i.e., the inner mitochondrial membrane (Fig. 1). Using this "mitoplast-attached mode," about two thirds of the patches were silent patches, suggesting a low density of the channel. After exchanging KCl in the pipette solution with an equal concentration of K-gluconate we observed a rectification of the current-voltage

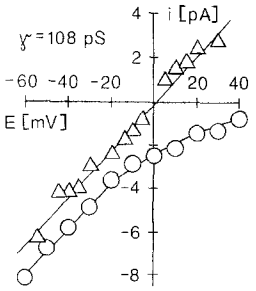

Fig. 2. Current voltage relation of the inner mitochondrial membrane anion channel, mitoplast-attached configuration. *Triangles:* 150 mM KCl solution in pipette and bath. *Circles.* Same bath solution, however, with 150 mM K-gluconate inside the pipette (i.e., outer membrane surface). There were no outward currents at positive holding potentials. Data were not from the same mitoplast as was shown in Fig. 1

(*i-E*) relation (Fig. 2, circles). As only Cl^- ions were removed, the missing inward anion current pointed to a Cl^- conductance of the channel. In three experiments with K_2SO_4 in pipette and bath we found an almost linear *i-E* relation with a mean γ of 56.3 ± 4.6 pS (*not shown*). We assume that in this case the *i-E* curves was linear because the mitoplast equilibrated to the changed anionic condition (Sorgato et al., 1987).

In addition to the 108-pS component of the current events there were infrequent channel openings at about 1/3, 1/2, and 2/3 of the main amplitude (Fig. 1). At least the 50% component seemed to be a substate because it was usually reached from the open state of the main 108-pS component, which would be difficult to understand if both states were distinct channels (*see* record at +50 mV in Fig. 1).

Comparing open times (two components of 0.75 and 57.9 msec) and closed times (two components of 0.17 and 0.98 msec) at +50 mV it was clear that the open state of the 108-pS channel was more occupied than the closed state (Fig. 3*A,B*). At positive potentials a very slow component (⩾100 msec) in the open time histogram (not visible in Fig. 3*A*) contributed strongly to the open probability, which was in one typical experiment in the range between 75 and 99% at positive potentials and declined to 40% at −20 mV.

Voltage Dependence

Closing of the channel is voltage dependent. As shown in the lower part of Fig. 1, at depolarizing potentials it was usually open, while at membrane potentials more negative than 0 mV there was a steep decrease in the probability of a channel being open. This is mainly due to the slow component of the open time histogram becoming faster. The time

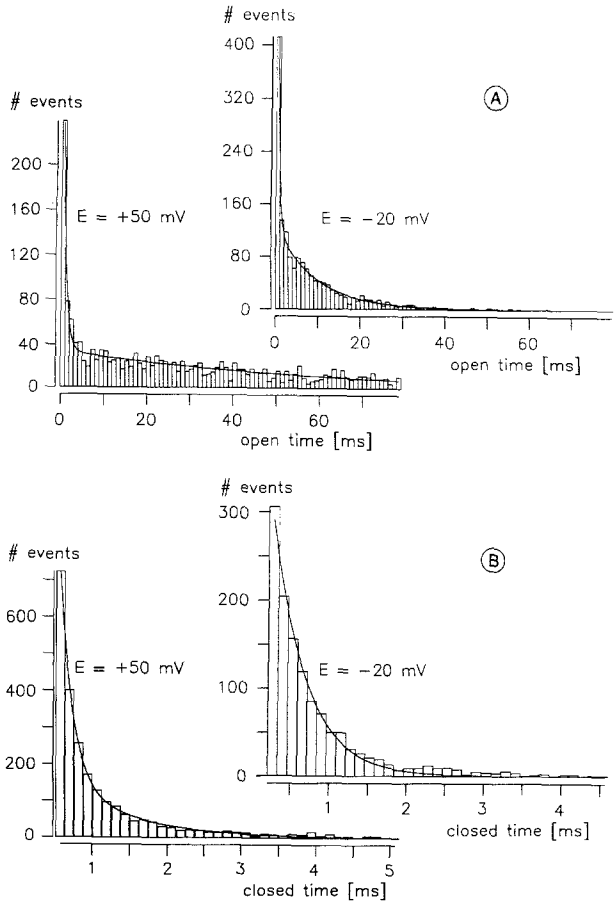

Fig. 3. Open- and closed time distribution at +50 mV (left) and −20 mV (right). (*A*) Open time histograms. Values were fitted best by two exponentials. Note the decreased contribution of the slow component at more negative potentials. +50 mV: τ_1 = 0.75 msec, τ_2 = 57.9 msec, 1770 events; −20 mV: τ_1 = 0.43 msec, τ_2 = 9.25 msec, 1527 events. (*B*) Closed time histograms. Two exponentials were used at +50 mV; at −20 mV a second exponential was not required. +50 mV: τ_1 = 0.17 msec, τ_2 = 0.98 msec, 2388 events; −20 mV: τ = 0.43 msec, 743 events

constant of the slow component of the open time decreased from 57.9 msec at +50 mV to 9.2 msec at −20 mV (Fig. 3*A*) and the current traces showed more flickering (Fig. 1, upper left). Changes in the closed time distribution were not so prominent (Fig. 3*B*). For recording current activity at negative potentials we kept the patch at +40 mV and then jumped to the new potential. The number of closings increased and after less than a minute the events vanished (Fig. 1).

Whole-Mitoplast Currents

Excising of patches from the tiny mitoplasts turned out to be impossible. Nevertheless, we wanted to test the influence of agonists as well as antagonists

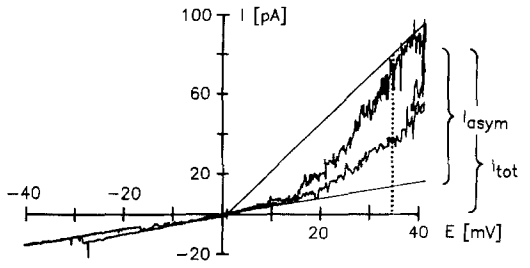

Fig. 4. Current across mitoplast membrane (I) *vs.* holding potential (E). Whole-mitoplast mode. E applied in ramps from 0 mV via +40 to −40 mV, and back to 0 mV. Current hysteresis loop at positive potentials due to short ramp duration (≈1 min for whole cycle) nicely illustrating voltage- and time dependence of the anion channel. Straight lines drawn by eye connecting (i) current in negative voltage range (anion channel closed) with current at reversal potential (0 pA) and (ii) current at reversal potential with maximum current during this ramp (neglecting brief spikes). Total current (I_{tot}) and asymmetrical part of current (I_{asym}) were measured at E = +35 mV (dotted line) as indicated by parentheses

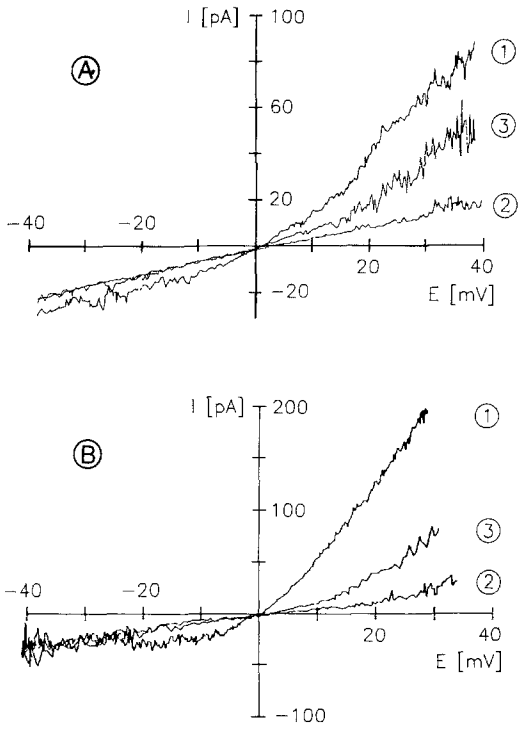

Fig. 5. Blocking effect of GDP (*A*) and GMP (*B*). Whole-mitoplast mode. *1:* Controls with 150 KCl solution in pipette and bath. *2:* Test solution with additional 20 μM nucleotide in bath. *3:* control after washing with 150 mM KCl solution. Stimulating ramps were as in Fig. 4 but lasting >2 min. Results have been collected in the Table

of the UCP. Thus we chose the whole-mitoplast mode (analogous to whole-cell mode) for reversible application of substances to the outer side of the inner mitochondrial membrane. Breaking the membrane at the mouth of the pipette was difficult. It usually happened spontaneously but with a relatively low success rate of about 1 : 15. Occasionally brief hyper- or depolarizing voltage pulses of up to 170 mV facilitated the transition from mitoplast-attached to whole-mitoplast mode. We had seen before that both the single channel current (triangles in Fig. 2) and the leakage current were linearly related to the holding potential and that the anion channel opened mainly at depolarizing holding potentials (lower part of Fig. 1). Thus asymmetry of the *I-E* curve was taken as a criterion for being in the whole-mitpolast mode (Sorgato et al., 1987). Increasing capacitive transients of the test current were additional criteria (*cf.* Materials and Methods). Using voltage ramps between −40 and +40 mV as a stimulus, the currents across the mitochondria inner membrane were recorded. To avoid large loops of hysteresis as shown in Fig. 4 the ramps had to be very slow (>2 min for a complete loop).

To calculate the number of channels in a mitochondrion, we assumed that most of the channels were open at +35 mV and that almost all of the channels were closed at negative membrane potentials. The maximum membrane current at +35 mV (I_{tot}) was the sum of the current flowing through anion channels and leakage current. For determination of this value the highest current value of the record and the reversal potential were connected. Due to the low open probability at negative potentials the *I-E* relation was almost linear in the hyperpolarizing range. Extrapolating this linear part to +35 mV gave a measure for leakage current. By subtracting it from I_{tot} the asymmetrical part (I_{asym}) which we suppose to be carried by the anion channel could be determined. I_{asym} was divided by 3.77 pA, the current flowing through a single channel at +35 mV, and we ended up with a mean number of 18.8 ± 8.1 (n = 28) channels per mitochondrion open at this holding potential.

Block by Adenosine- and Guanosine Nucleotides

In order to test for a relation between the UCP pathway and the anion channel we tried to block the 108-pS channel by ADP, ATP, GDP, and GTP (20 μmol). These substances are known to be responsible for coupling of respiration and ATP synthesis in brown adipocyte mitochondria (Klingenberg, 1984). In the depolarizing voltage range Fig. 5*A* shows a current (I_{asym}) clearly reduced by GDP. Similar results were obtained with the other di- as well as trinucleotides but, to our surprise, also with GMP, a mononucleo-

Table. Decrease of total membrane current (δI_{tot}) and of current due to anion channel (δI_{asym}) under the influence of 20 μM purine di- and trinucleotides (in % ± SD)[a]

	δI_{tot} (%)	δI_{asym} (%)	n
ATP	46.5 ± 12.8	52.6 ± 9.1	3
ADP	77.3 ± 6.2	83.6 ± 7.5	3
GTP	62.6 ± 11.2	68.3 ± 7.2	4
GDP	43.9 ± 23.0	52.9 ± 24.1	6
GMP	74.9 ± 14.8	81.2 ± 14.8	3
cGMP	6.9 ± 0.4	8.1 ± 2.6	2

[a] Currents were determined as explained in Fig. 4.

tide (Fig. 5*B*). All effects were partly reversible. In control experiments cyclic GMP (cGMP) or plain phosphate (20 μM each) did not show a significant effect. It is unlikely that the influence of the low nucleotide concentrations was due to their ability to bind free Ca^{2+}. In two experiments in the mitoplast-attached mode we saw normal single channel activity with KCl solutions containing 2 mM EGTA and no added Ca^{2+}. Data for nucleotides are summarized in the Table. The effect on the total current at +35 mV (I_{tot}) was always smaller than on I_{asym}, indicating a stronger effect of the nucleotides on the anion channel than on the leakage current.

As occasionally some channels were still open at hyperpolarizing potentials (Fig. 4), it was difficult to determine a possible nucleotide effect on the symmetrical part of the membrane current with sufficient accuracy. On the other hand, it can be calculated that proton currents carried by the UCP are probably very small. From the proton conductance of a single UCP of 10 ions/min/mV (Nicholls et al., 1986, corresponding to 0.027 fS) and a number of about 10^5 to 3×10^5 UCP dimers (= pores) per mitochondrion (J. Rafael, *personal communication*), one can estimate a total conductance change after complete uncoupling of 2.7 pS which is close to the limit of detection with our method. Thus if the proton current was visible, it should have shown up in the hyperpolarizing range of the *I-E* curve. In three screening experiments we saw a decrease of 122, 135, and 34 pS in membrane current when switching from our 150 mM KCl bath solution without GDP to one containing 20 μM GDP. This effect was reversed or even overcompensated by 269, 127, and 81 pS, respectively, when 50 μM palmitic acid were subsequently added.

Discussion

Mitochondria of brown adipocytes are larger than most other mitochondria. So fortunately it was unnecessary to use cuprizone-fed animals as Sorgato et al. (1987) were forced to do. Our results show that there is a voltage-dependent anion-selective channel of 108 pS in the inner mitochondrial membrane of brown adipocytes. Sorgato et al. (1987, 1989) probably found the same type of channel (107 pS) in liver cell and heart cell mitochondria of mouse and ox. They and Selwyn (1987) questioned whether this channel could have something to do with the UCP and NST. However, uncoupling of mitochondria is thought to be a unique property of brown adipose tissue (Nicholls et al., 1986).

The similarities between the anion channel we found and the channel in liver and heart cell mitochondria are that both channels have anion selectivity, the same single channel conductance (107 *vs.* 108 pS), and comparable open- and closed time distributions. The fast component of the closed time distribution of Sorgato et al. (1987) is very close to our slow component (1.9 msec at +60 mV *vs.* 1.0 msec at +50 mV). A faster component may have escaped their detection due to the lower filter frequency Sorgato et al. (1987) used. On the other hand, our current traces show additional closed times which could contribute to a very slow process. Their single open time constant was between both of our open time constants. In order to further prove the comparability of our results from brown adipose tissue and theirs from a liver cell preparation, we also recorded from rat liver cell mitoplasts. No obvious difference was observed between our experiments on mitoplasts of both organs.

Whole-mitoplast currents were used to test the effect of nucleotides and to calculate a mean number of channels per mitochondrion. The number of 18.8 is a lower limit of the number of channels per mitochondrion since it is possible that in the limited time and potential range of our ramps we did not catch a moment with all channels open at the same time. Due to the voltage dependence of the anion channel the likelihood of all the channels being opened should increase with more depolarizing potential. On the other hand, the lifetime of a whole-mitoplast configuration decreased enormously with decreasing or increasing holding potential. The chosen potential range of −40 to +40 mV seemed to be reasonable compromise.

The K_D of di- and trinucleotide block was determined to be about 0.8–5.0 μM for UCP (Klingenberg, 1984). In our experiments we saw a 53–84% block by a concentration of 20 μM. The main difference between anion-channel block and UCP inhibition was that GMP blocked the 108 pS channel as well as the other di- or trinucleotides while the K_D of GMP for isolated brown fat cell mitochondria was 110 μM (Lin & Klingenberg, 1982). The differences in the concentration dependence of nucleotide block

could indicate that the UCP and the halide anion channel are distinct (Klingenberg & Winkler, 1985; Kopecky et al., 1984; but *see* Nicholls et al., 1986, for criticism). Nicholls (1974) found a halide anion conductance associated with the UCP. He suggested that it might be an additional property of the molecule with a predicted single channel conductance of only 0.027 fS. Thus the anion conductance we described (108 pS) would be another so-far unknown anion channel in brown fat and also in other mitochondria.

With a mean number of 18.8 anion channels per mitochondrion and a single channel conductance of 108 pS, three orders of magnitude higher as compared with the UCP system regulating nonshivering thermogenesis, this would be a powerful system. However, we do not think that the 108-pS channel has a high open probability in the physiological potential range. But as activated mitochondria, especially short-circuited brown fat cell mitochondria, undergo large volume changes (Cannon & Nedergaard, 1987), there may be a demand for these channels in volume regulation. This would explain why we found them active in our osmotically swollen mitoplasts where some regulatory factors may be absent (Kinnally, Campo & Tedeschi, 1989). But still it seems extremely important that a nucleotide binding site in the inner mitochondrial membrane other than the UCP or the ATP/ADP translocator seemed to be present.

There are five arguments that the anion conductance described here for brown adipocyte mitochondria has to be separated from the functional proton shunt responsible for NST: (i) The functional proton shunt through the UCP is thought to be a unique property of brown adipocytes, whereas the anion channel has been found in liver and heart cell mitochondria as well (Sorgato et al. 1987, 1989; Kinnally et al., 1989). (ii) The proton conductance and the UCP-related halide anion conductance in the absence of nucleotides are similar and were predicted to be less than 0.1 fS (Nicholls et al., 1986). This is many orders of magnitude smaller than the 108 pS described here. (iii) For the UCP the K_D of GMP is about 44 times bigger than that of GDP (Lin & Klingenberg, 1982). In our experiments the blocking effect of GMP was about comparable to the di- and trinucleotides. (iv) The proton conductance of the UCP is known to be strongly pH dependent (Malan & Mioskowski, 1988), while the anion conductance is not (Sorgato et al., 1987). (v) The voltage dependence of the anion conductance makes it likely that it is closed at depolarizing potentials which are thought to permit NST. Thus we are left with the conclusion that the anion channel described here is not the functional proton shunt responsible for NST.

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