Journal of Bioenergetics and Biomembranes, Vol. 28, No. 2, 1996

Anion Channels of the Inner Membrane of Mammalian and Yeast Mitochondria

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Received May 21, 1995; revised July 6, 1995

The inner membrane of yeast and mammalian mitochondria has been studied *in situ* with a patch clamp electrode. Anion channels were found in both cases, although their behavior and regulation are different. In mammalian mitochondria, the principal channel is of around 100 pS conductance and opens mainly under depolarized membrane potentials. As no physiological compound able to alter its peculiar voltage dependence has yet been found, it is proposed that this channel may serve as a safeguard mechanism for recharging the mitochondrial membrane potential. Two other anion channels, each with a distinct conductance (one of approx. 45 pS, the second of at least a tenfold higher value) and kinetics are harbored in the yeast inner membrane. Matrix ATP was found to interact with both, but with a different mechanism. It is proposed that the 45 pS channel may be involved in the homeostatic mechanism of mitochondrial volume.

KEY WORDS: Channel; patch clamp; mitochondria; Cl_{ATP} channels; inner mitochondrial membrane.

INTRODUCTION

The *in situ* electrophysiological analysis of the inner membrane of mitochondria (IMM) with a patch clamp electrode has undoubtedly established that this membrane harbors a variety of high-conductance pathways (e.g., Sorgato and Moran, 1993; Zoratti and Szabò, 1994). However, it is also true that their role is far from being defined, primarily for two reasons. The first is somehow conceptual, in that, at first instance, the high flux of ions mediated by channels may appear in conflict with the current notion of mitochondrial bioenergetics. The second reason is more practical: with few exceptions (Diwan *et al.*, 1990; Paucek *et al.*, 1992; Paucek *et al.*, 1995), a biochemical approach to the isolation and reconstitution of the channels described electrophysiologically is largely lacking.

This paper is devoted primarily to the description of the functional properties of the channels best characterized in our laboratory with the patch clamp technique, i.e., the anion channels of the inner membrane (IM) of mammalian and yeast mitochondria. Based on our data and on the information provided by other laboratories, a tentative hypothesis of the role of some of them will be put forward.

THE MAMMALIAN mCS CHANNEL

Electric Features

Several laboratories have described the mitochondrial centum picoSiemen (mCS) channel in the native IMM isolated from a variety of mammalian tissues (Sorgato *et al.*, 1987, 1989; Petronilli *et al.*, 1989; Inoue *et al.*, 1991; Kinnally *et al.*, 1991; Klitsch and Siemen, 1991). The distinctive features of the mCS channel (extensively reviewed in Sorgato and Moran, 1993) are the following:

—it has a conductance close to 100 pS (in symmetrical 150 mM KCl);

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0145-479X/96/0400-0125$09.50/0

— it has an open probability (P_o) which increases with depolarization;
— it is more permeable to anions (P_{Cl}/P_K = 4.5);
— it is mainly responsible for the macroscopic current of the entire IMM;
— it is present in approx. 50–300 copies per single mitoplast.

Regulation by Physiological Compounds

Regulation of the mCS by physiological molecules is unknown or at least is not yet satisfactorily established. Variations of H^+ concentration on either side of an IM patch and of Mg^{2+}, for example, are unable to alter the channel behavior (Sorgato *et al.*, 1987; Szabò and Zoratti, 1992). On the other hand, some observations that adenosine (and guanosine) nucleotides (Klitsch and Siemen, 1991) and Ca^{2+} (Kinnally *et al.*, 1991) may act as negative modulators do not find complete agreement in the literature. Indeed, Inoue *et al.* (1991) and Szabò and Zoratti (1992) were unable to detect modifications of the channel after addition of ATP or ADP. As for Ca^{2+}, which was reported to inhibit activation of, or reduce, channel activity in mitoplasts if present, respectively, in the cytoplasmic or matrix side of the IM (Kinnally *et al.*, 1991), the selective presence or absence of the ion did not yield similar results in other laboratories (Sorgato *et al.*, 1987, 1989; Petronilli *et al.*, 1989; Klitsch and Siemen, 1991; Szabò and Zoratti, 1992). In the light of the crucial importance of Ca^{2+} in promoting cytoplasmic as well as mitochondrial metabolic pathways, we have recently reinvestigated its effect by following mCS activity in mitoplasts specifically prepared with or without Ca^{2+} chelators (Kinnally *et al.*, 1991) and/or by modifying the concentration of Ca^{2+} in the medium facing excised patches. Once again, in our hands, the various combinations produced no difference in the detection of channel activity (unpublished observations). At face value, it is hard to find a satisfactory explanation for these discrepant results. A plausible cause could be a regulator conferring Ca^{2+} sensitivity, being left or removed depending on the preparation, although the step *per se* of the removal of the outer membrane to obtain mitoplasts (by French press or osmotic shock) seems not to be implicated (Kinnally *et al.*, 1991).

Hypothetical Function

Several possible roles for mCS channels have been postulated, where either they work independently (Sorgato and Moran, 1993) or are part of complex super molecular structures residing preferentially where the two mitochondrial membranes are closely apposed (Kinnally *et al.*, 1993). None of these hypotheses has, however, been proven definitively. Also, no agent has yet been described to be capable of reversing the voltage dependence of the channel, i.e., to induce the opening at those high negative voltages sustained by mitochondria. Thus, at least for the time being, it appears compulsory to accept the notion that mCS channels function only when mitochondria are under depolarized (or quasi-depolarized) conditions. The question that naturally follows is whether there is any positive role for them under these particular circumstances.

According to our understanding, the only role could be that of a safeguard mechanism. Fundamental to such a hypothesis are two experimentally proven features and one assumption. The features are the anion selectivity and the higher P_o at positive or low negative values of the membrane potential. On the other hand, the assumption visualizes that at physiological potentials (in the range of −150 mV, −180 mV) the anion capable of permeating the channel can, in spite of the closed state of the channel, reach quasi-electrochemical equilibrium across the IM by passive (albeit slow) movements. If this were the case, then a drop of the potential below −100 mV or so (for a transient failure of the respiratory chain proton pump activity, for example, or for an extrinsic cause) would favor the opening of some mCS channels and the consequent extremely rapid anion entry into the matrix to establish the equilibrium potential for that ion. In essence, the uncompensated charge movement across a membrane with minimal electrical capacity, as is characteristic of the IMM, would build up a substantial diffusion potential, negative inside, which would, in turn, shut off the channels. But then steady-state conditions could be reestablished by the activity of the respiratory chain. With respect to which anion might be involved in this putative function, the best candidate appears to be chloride on the basis of its (mM) cytoplasmic concentration and of its ability to permeate mCS channels, but the search for other anions able to act similarly is perhaps desirable.

THE TWO CL_{ATP} YEAST CHANNELS

The analysis of the IM of yeast mitochondria has been carried out recently in our laboratory (Ballarin and Sorgato, 1995a), by use of mitoplasts obtained by subjecting fresh mitochondria to milder hypotonic steps than those currently employed with liver mitochondria (Ballarin and Sorgato, 1995b). Patch clamp experiments have revealed the presence of a low and of a high conducting channel, which have in common a preferential anion selectivity (P_{Cl}/P_K around 3–4) but otherwise have different electric features, as detailed below.

Electric features of the small channel:
— it has a conductance of ~45 pS, slightly decreasing at negative voltages;
— the P_o is low at all voltages (slightly increasing at negative voltages);
Electric features of the large channel:
— it has a conductance which markedly rectifies at positive voltages (~400 pS at −40 mV; ~800 pS at 40 mV);
— the P_o is voltage dependent (much higher at positive voltages).

Figure 1 shows traces of a patch where another distinctive feature of the small channel is evident (i.e., the higher flickering at positive potentials). On the other hand, traces of the large conductance contained in Fig. 2 reveal all the predominant electric characteristics of this channel. Either channel maintained its behavior irrespective of the configuration of the patch (cell attached or excised). Identical activities were found in mitoplasts obtained from a strain lacking the gene encoding for the most abundant channel in the outer mitochondrial membrane, the voltage-dependent anion channel (VDAC).

Small and large conductances appeared frequently together in the same patch, which shows that both channels belong to the same (inner) membrane. No other type of channel was found although the background noise might have shielded conductances lower than 15–20 pS. Lack of an activity resembling the mCS conductance came as surprising, in view of the

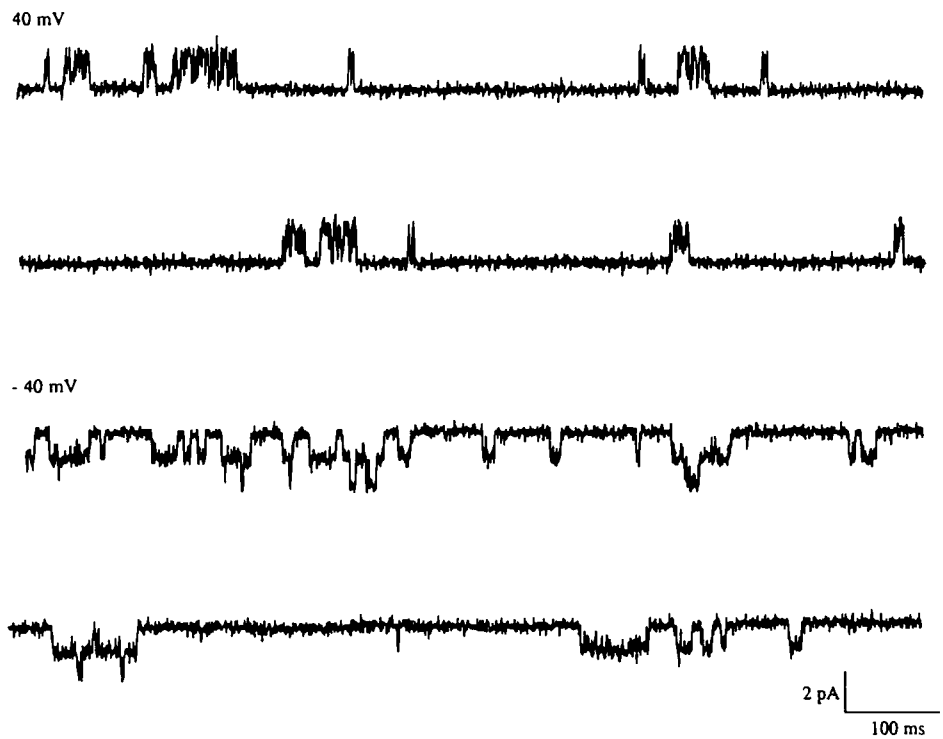

Fig. 1. Electric behavior of the low-conductance anion channel of the yeast IMM. Traces were recorded from an inside out patch (Hamill *et al.*, 1981) of a mitoplast obtained from the wild type HR125-2B *Saccharomyces cerevisiae* strain and filtered at 1 kHz. At least four channels were present, which accounts for the high number of events, especially at negative voltages (see text). Gain, 50 mV/pA.

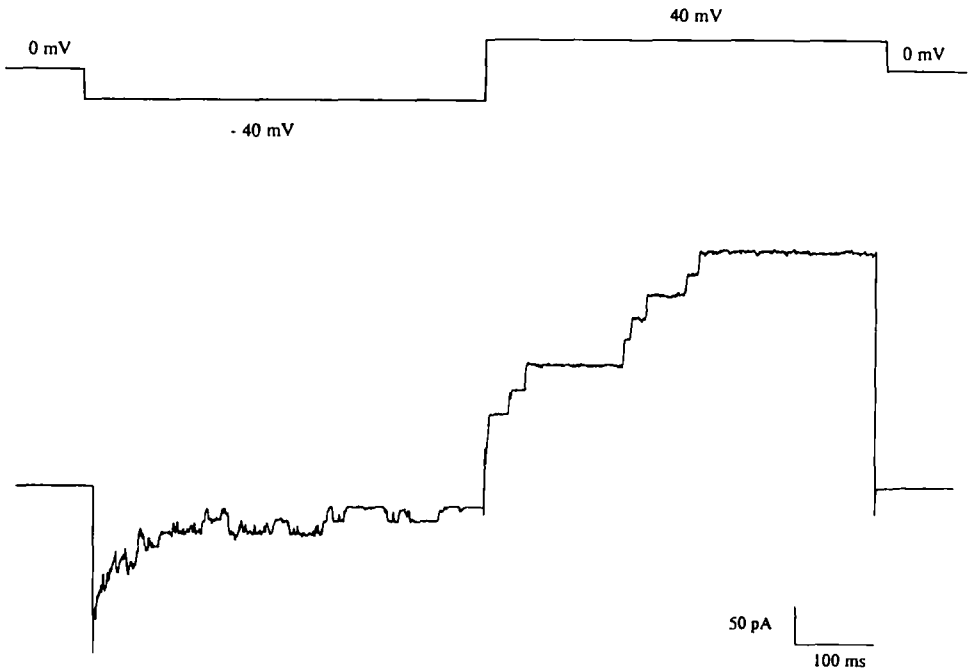

Fig. 2. Electric behavior of the high-conductance anion channel of the yeast IMM. Traces were recorded after applying voltage pulses (see figure) to a mitoplast attached patch obtained from the wild type HR125-2B *Saccharomyces cerevisiae* strain. The patch contained at least nine channels. Note the fast kinetics of the closing events and the slow kinetics of the opening processes and the different single-channel conductance at positive and negative voltages. Filter, 1 kHz. Gain, 10 mV/pA.

same physiology pertaining to mitochondria of low and high eukaryotes and of the relative ease of detecting mCS channels in the mammalian membrane (see also Lohret and Kinnally, 1995). Likewise, there was absence of the multiconductance channel (MCC) characterized in mammals by some laboratories (for a review see Sorgato and Moran, 1993) and apparently present also in the wild type yeast IM with very similar features (Lohret and Kinnally, 1995) (but see Szabó *et al.*, 1995). Parenthetically, there is no doubt that the large conductance described by us in yeast is sharply distinctive from that assigned by Lohret and Kinnally to MCC activity. In fact the latter displays peak conductances of 1–1.5 nS (more frequently at negative potentials) and a wide range of sublevels (particularly at positive voltages), is more permeable to cations, and the P_o is higher at negative voltages. All these features are clearly diverging from those listed above. Moreover, in spite of the apparent quasi-similar behavior under some experimental conditions (e.g., in cell-attached patches), other properties of the large channel, reported by Szabó *et al.* (1995) in VDAC-less yeast mitoplasts, render unlikely, at least for the time being, that this channel may coincide with that described by us.

Regulation by Physiological Compounds

Like the mammalian mCS conductance, both yeast channels are insensitive to matrix Ca^{2+} and Mg^{2+}. Conversely, there is a definite interaction with physiological concentrations of ATP added to the matrix side of the IM (Fig. 3). The effect of the nucleotide depends, however, on the channel: the small one gets completely inhibited (Fig. 3A) (IC_{50} for ATP = 0.24 mM) whereas the large channel loses the voltage dependence and remains permanently open at voltages of either sign (Fig. 3B) (Ballarin and Sorgato, 1995a).

Hypothetical Function

The features of the Cl_{ATP} channels found in yeast are at variance with what is currently known of other anionic conductances studied electrophysiologically *in*

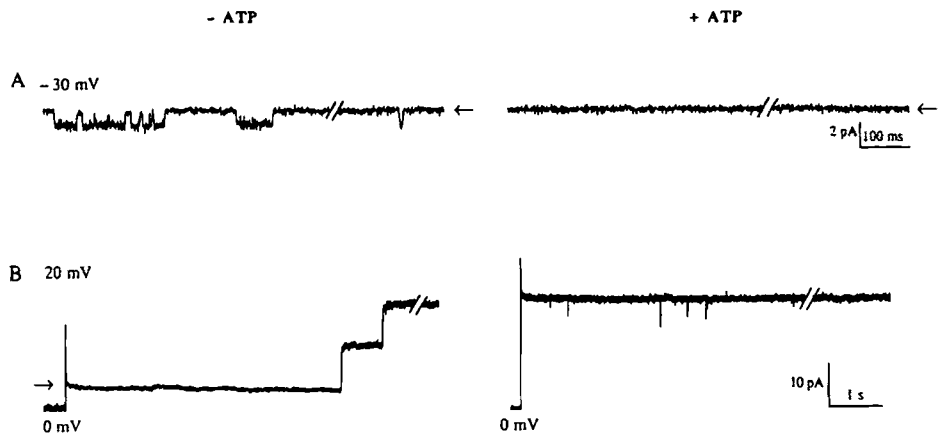

Fig. 3. Effect of ATP on yeast IMM anion channels. Continuous recordings from mitoplast excised patches showing activity of the low conductance (A) and large conductance (B) channel, before and after the addition of ATP (~1 mM). In B, the current transition from 0 mV is also shown. The different effect of ATP on the two channels was independent of the sign of the applied voltage. Arrows indicate the closed state, and interruptions indicate a time span of several sec. In either case the wild type HR125-2B *Saccharomyces cerevisiae* strain was used. Filter, 0.7 kHz. Gain, 50 mV/pA (A) and 10 mV/pA (B).

situ (Sorgato and Moran, 1993; Antonenko *et al.*, 1994), in planar bilayers (Hayman *et al.*, 1993), or in intact mitochondria by means of the swelling technique (Beavis, 1992; Manon and Guèrin, 1993; Guèrin *et al.*, 1994). For example, IMAC (inner membrane anion channel), a protein involved in anion transport across the inner membrane, is known to get activated by a decreased concentration of free matrix Mg^{2+} (Beavis, 1992; Beavis *et al.*, 1993; Antonenko *et al.*, 1991, 1994). In the case of the two yeast Cl_{ATP} channels, on the contrary, their activity in excised patches was insensitive to the addition of mM Mg^{2+} (Ballarin and Sorgato, 1995a). Also, given the lack of effect of bongkrekic acid (Ballarin and Sorgato, 1995a), it is unlikely that one of them is involved in translocation of adenine nucleotides. Other possibilities have then to be considered by evaluating, for example, if a role attributed to Cl^- channels of the plasma membrane may, by analogy, be transposed to the IM, or by assessing if, on the basis of the current knowledge of mitochondrial physiology, there is a necessity for the IM to harbor an anion channel with the functional behavior described by us.

Plasma membrane Cl^- channels have been implicated in diversified physiological functions (Pusch and Jentsch, 1994). For the present discussion, it is worth recalling their involvement (in association with movement of cations) in the phenomenon of regulatory volume decrease (RVD) of cells exposed to hypotonic media (Sarkadi and Parker, 1991).

In the case of mitochondria, it is established that a prime hazard for the organelle is the influx of cations, particularly of potassium, driven by the high cytosolic concentration and the high membrane potential, negative inside. A parallel influx of anions (via an electroneutral H^+ symport mechanism) would then lead to water uptake, swelling, and eventually lysis of mitochondria. This dangerous event is, however, occurring *in vivo* only under severe circumstances, because mitochondrial integrity is largely preserved by compensation of potassium uptake [initially viewed to occur through leak pathways (Garlid, 1988)] with the activity of the electroneutral K^+/H^+ antiporter (Garlid, 1988, 1994), finely modulated by pH and Mg^{2+}. It follows that the rate of K^+ and H^+ exchange is crucial for the control of matrix volume: an exchange rate lower than K^+ influx will lead to swelling, a higher rate to shrinking. Another key point of volume homeostasis has been recognized in the inner membrane anion channel (IMAC). This protein transports a variety of unrelated anions (including ATP), is modulated similarly to the K^+/H^+ antiporter, and is thought to be important especially for volume contraction under particularly severe physiopathological stress (Garlid and Beavis, 1986; Beavis, 1992).

Recent findings, however, even if they do not contradict the above initial picture, nonetheless suggest that other pathways for ions might be involved in the control of mitochondrial volume. The postulated existence of a K^+ uniport (for a review see Garlid,

1994) was finally proven, at the single-channel level, by patch clamping bare inner membranes (Inoue *et al.*, 1991), and further supported by experiments with proteoliposomes (Paucek *et al.*, 1992) and intact mitochondria (Beavis *et al.*, 1993; Manon and Guèrin, 1993). Importantly, in all cases inhibition by adenine nucleotides (ATP, ADP, and AMP) was found. The functional characteristics of this K_{ATP} channel find a match in the lower-conductance anion channel of the yeast IM in that both display a low (single-channel) conductance, are essentially voltage-independent, and are inhibited by ATP. It is therefore highly tempting to associate the channel-mediated K^+ and Cl^- fluxes with a common physiological role, i.e., in KCl uptake and matrix swelling (Beavis *et al.*, 1993). Important details, such as the identification of the molecule(s) able to relieve the proteins from the adenine nucleotide inhibition, have still to be elucidated. Such possibility must exist as it appears nonsensical that inactive proteins are being harbored in the inner membrane of mitochondria. On the other hand, the very potent inhibition by physiological ATP argues in favor of activation of channels only under particularly dangerous conditions when powerful means of net salt movement are warranted.

ACKNOWLEDGMENTS

The work was supported by grants of the Consiglio Nazionale delle Ricerche and of the Ministero della Ricerca Scientifica e Tecnologica of Italy. The authors thank Dr. M. Forte, Oregon Health Science University, Portland, Oregon, for providing them with the HR125-2B and VDAC-less *Saccharomyces cerevisiae* strains.

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