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Electrophysiological Techniques for Mitochondrial Channels

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1. Introduction

The patch-clamp technique has revolutionized studies of ion channels in a wide variety of cells. Its development in the late 1970s and early 1980s made it possible to analyze the currents of single ion channels. However, electrophysiological recordings of these proteins were limited to the plasma-membrane of living cells: the small size and the double-membrane organization of some cell organelles and the reduced stability of intracellular membranes precluded an implementation of this technique to investigate the properties of channels located in mitochondria, chloroplasts, the endoplasmic reticulum or lysosomes. For the last 25 years great effort has been made to implement the patch-clamp technique besides the planar lipid bilayer approach on these organelles. In this mini-review we focus on the developments and applications of the patch-clamp technique on mitochondrial and other cytosolic membranes and discuss the challenges of different, innovative approaches.

2. First attempts

The patch-clamp technique developed by Neher and Sakmann in 1976 as an effective refinement of the voltage-clamp-assay proved the function of single ion channels in fundamental cellular processes (Neher and Sakmann, 1976). The effectiveness of this technique to study plasma-membrane localized channels soon became of interest to characterize putative ion channels in intra-cellular membranes. Special attention was paid to mitochondria and their main function to provide the cell with energy in the form of ATP. Still in 1976, members from the lab of Alan Finkelstein inserted mitochondrial membranes into planar lipid bilayers using a Triton X-100 extract of rat liver mitochondria (Schein *et al.*, 1976). Single channels were directly detected by elevated ion permeability through the bilayer. This innovative work and further studies in the following years (Roos *et al.*, 1982) provided a good deal of information on the probable behavior of the voltage-dependent anion channel (VDAC, "mitochondrial porin"). However, the permeability properties of the intact outer mitochondrial membrane (OMM) remained hidden. In 1987, Tedeschi and colleagues managed to fill gaps in information about the electrical characterization of the OMM (Tedeschi *et al.*, 1987): they successfully introduced the patch-clamp technique on

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outer membrane vesicles isolated from mitochondria of the fungus *Neurospora crassa* and giant mitochondria isolated from cuprizone-fed mice. The cupper chelator Cuprizone (biscyclohexanone oxaldihydrazone) had been shown to induce an increase of the size of mitochondria more than twofold (Flatmark *et al.*, 1980). Their results confirmed the presence of the VDAC, which facilitates the exchange of ions and molecules up to 5 kDa. Kathleen W. Kinnally, a pioneer in the field of mitochondrial electrophysiology, and co-workers continued their work on channels of the OMM. Among other findings they presented first studies on the kinetics of conductance changes induced by negative voltages and speculated on the multimerization of the high-conductance channel in the OMM (Kinnally *et al.*, 1989b). It took more than 30 years after its initial discovery, to unravel the structure of the VDAC channel by NMR spectroscopy and X-ray crystallographic techniques (Bayrhuber *et al.*, 2008;Hiller *et al.*, 2008;Ujwal *et al.*, 2008).

Apart from the progress that had been made on channels of the OMM, the importance of putative channels in the inner mitochondrial membrane (IMM) was neglected. Until the late eighties it was widely believed that the IMM was unlikely to contain any channels. The small size and the double-membrane structure of mitochondria had prohibited researchers from using the patch-clamp technique on the IMM. This prevailing opinion was finally disproved by the group of Walter Stühmer in 1987 by patch-clamp recordings of the inner mitochondrial membranes (IMM) (Sorgato *et al.*, 1987). They circumvented the restriction in size and the double-membraned organization by removing the OMM using a swelling-shrinking-sonication procedure from mitochondria isolated from cuprizone-fed mice. This led to the formation of vesicles termed mitoplasts with diameters of 3-6 µm. Measurements in whole mitoplast configuration as well as single channel recordings resulted in the identification of a voltage-dependent, anion permeable channel. The properties of this 107 pS channel, which was later found to correspond to the Inner Membrane Anion channel (IMAC) (Borecky *et al.*, 1997;Schonfeld *et al.*, 2004), were reported to be remarkably different from those of the VDAC of the OMM with a calculated mean conductance of 480 pS (Roos *et al.*, 1982).

3. Pros and cons of electrophysiological methods

In order to characterize the electrophysiological properties of single channels, two methods are commonly used: a voltage-clamp assay using planar lipid bilayers and the glass-pipette based patch-clamp method. The former one is based on the incorporation of purified membrane vesicles into the bilayer. The method allows studying channels, which are not easily accessible for the patch-clamp technique. Thus, the IMM but also other intracellular organelles including endosomes, lysosomes or the Golgi-apparatus may be analyzed. Furthermore this method is capable of detecting changes in the channel properties upon modulation by other proteins or lipids, i.e. variations in the lipid or protein composition of the bilayer. The major advantage of the patch-clamp method is the suitability to study channels in their natural environment, as the isolation process for the bilayer method may be harmful for the channel. Exemplarily, the recorded VDAC currents through single channels were 350 pS in patch-clamp recordings of whole mitochondria (Sorgato *et al.*, 1987), while a single channel conductance of 480 pS was found for the same channel after reconstitution in lipid bilayers (Roos *et al.*, 1982). The differences in the conductance might originate from a distinct membrane environment. However, the purity of the vesicle preparation is crucial due to the different fusion probability of vesicles from various membranes. This problem can be partly solved by using vesicles lacking any other

contaminating membranes. Disadvantages of the patch-clamp technique should be mentioned at this point as well: first, mitochondria and possibly other cell organelles are in close contact with membranes of the endoplasmic reticulum, forming so-called "mitochondria associated membranes" (Garcia-Perez *et al.*, 2008). It might be difficult to completely remove these membranes. Second, some membranes, e.g. mitochondrial Cristae tubules, might not be accessible for the glass pipette (Zick *et al.*, 2009). Thus, the patch clamp technique might fail to detect some important ion channel currents.

4. Mitochondrial electrophysiology in the nineties

A report of the group of T. Higuti in 1991 can be considered another milestone in mitochondrial electrophysiology: they characterized a highly K^+ selective, low conductance channel in the IMM, using mitochondria isolated from fresh liver of rats (Inoue *et al.*, 1991). To date the activity identified as corresponding to that of the ATP-sensitive K^+ channel (mito KATP or mtK_{ATP}) remains a matter of controversy. The molecular composition of the mito KATP has not been unraveled and some reports challenge the presence of this channel in the IMM (Brustovetsky *et al.*, 2005;Das *et al.*, 2003), while most studies support its existence (Dahlem *et al.*, 2004;Zhang *et al.*, 2001). However, the chosen patch-clamp strategy for the IMM appeared promising: To circumvent the double-membrane structure and small size of mitochondria, they removed the OMM by digitonin treatment and fused the generated giant mitoplasts using a low-pH solution containing Ca^{2+}. This method does not require additional lipids necessary for the vesicle fusion process that could influence the channel in its native environment. In addition the achieved size of the fused mitoplasts of up to 15 μm allows for a robust giga-seal formation with the patch-pipette tip.

A first report on the mitochondrial multiconductance channel by Kinnally and co-workers in 1989 (Kinnally *et al.*, 1989a) opened up a new chapter in mitochondrial electrophysiology, which has not lost its relevance. The MCC, also termed mitochondrial megachannel, was later described as the mitochondrial permeability transition pore (MPT pore or MPTP), which is formed under certain pathological conditions (Lemasters *et al.*, 2009). At that time, first amphiphilic drugs were found to affect the mitochondrial permeability (Beavis and Powers, 1989;Beavis, 1989), which was regarded as a prerequisite for the study of channels in the IMM (Antonenko *et al.*, 1991;Gunter and Pfeiffer, 1990). Electrophysiological data on mitoplasts created in the lab of Mario Zoratti proposed the MCC may comprise VDAC molecules (Szabo *et al.*, 1993). Further biochemical investigations by Andrew Halestrap et al. and electrophysiology studies performed in the Kinnally-lab identified the adenine nucleotide translocase (ANT) as the central unit forming the MPTP (Halestrap *et al.*, 1998;Lohret *et al.*, 1996). Although these findings have been challenged by studies published only a few years ago (Baines *et al.*, 2007;Kokoszka *et al.*, 2004), the efforts of these few pioneers during the nineties accelerated the progress for a better understanding of the complex behavior of mitochondrial channels.

In contrast to the work of Inoue et al, 1991 (Inoue *et al.*, 1991) on the mito KATP channel, most researchers did not work on fused mitoplasts, but continued to directly patch-clamp whole mitoplasts prepared from mammalian cell lines or tissue. These included a glioma cell line (Siemen *et al.*, 1999), an osteosarcoma cell line (Murphy *et al.*, 1998), rat liver tissue (Szabo *et al.*, 1992) or brown adipose tissue from hamsters (Borecky *et al.*, 1997). However, only mitochondria isolated from a limited number of mammalian and other eukaryotic cells

formed mitoplasts with an adequate size. Thus, most IMM channel activities were recorded after reconstitution into proteoliposomes or giant liposomes (Lohret *et al.*, 1996;Lohret *et al.*, 1997;Paliwal *et al.*, 1992). This holds especially true for studies of yeast mitochondrial channels. The small size of mitoplasts from this organism (0.3-1.5 μm), either isolated by osmotic swelling or using the French press method, makes the patch clamp technique very difficult. The advantages of the model-system yeast, e.g. fast growth, easy handling and especially the possibility of a fast gene knock-out, justify the technically challenging and laborious reconstitution process. Still, the purity of the used membrane fractions and a potential influence of externally added lipids for the generation of giant liposomes must be taken into account.

5. Mitochondrial electrophysiology over the past 10 years

We may start with an outstanding finding published in 2004 (Kirichok *et al.*, 2004). Yuriy Kirichok *et al*. from the lab of David Clapham described the properties of a channel consistent with the predicted mitochondrial Calcium uniporter (miCa). The experiments were accomplished by patch-clamp recordings of whole mitoplasts without any additional fusion or reconstitution processes. An interesting feature of the chosen COS-7 cell-line is that intact mitoplasts of 2-5 μm size could be prepared. For that purpose mitochondria were isolated by differential centrifugation and mitoplasts were formed by osmotic swelling. Interestingly, no additional steps to remove the OMM had been performed, which enhances the risk that remnants of the OMM or the tightly attached ER-membrane falsify the results. According to the authors such artifacts had been excluded as these structures appeared as black spots on the transparent vesicles. Taken together extreme care has to be taken and numerous technical problems have to be solved when performing such experiments. Very recently, the molecular identity of the miCa has been clarified: a channel protein termed MCU and a regulating protein named MICU1 have been reported to fulfill the requirements for the long searched mitochondrial Calcium uniporter (De *et al.*, 2011;Perocchi *et al.*, 2010).

Inspired by the work of these pioneers our group started electrophysiological studies on mitochondrial Magnesium transport. In 2003-2006 we had shown, using Magnesium selective fluorescent dyes, that the IMM localized protein Mrs2 is a major player for the influx of Magnesium into yeast mitochondria (Kolisek *et al.*, 2003;Weghuber *et al.*, 2006). At that time we could not exclude that Mrs2p forms a transporter rather than a high-conductance channel. To answer this question we generated mitoplasts using osmotic swelling or the French press method. However, consistent with the work performed in the Kinnally lab in the 90ies, the small size of yeast < 1 μm did not allow for effective patch-clamp recordings. Thus we decided to fuse IMM with lipid vesicles: submitochondrial particles (SMPs) (Froschauer *et al.*, 2009) were mixed with asolectin vesicles. According to the report of Criado and Keller (Criado and Keller, 1987) in 1987 we finally succeeded in generating giant vesicles (5-50 μm in size) with reconstituted IMM fractions from yeast overexpressing or having *MRS2* disrupted. The used method is characterized by dehydration-rehydration cycles and has also been successfully used by other groups working on yeast mitochondrial channels (Lohret *et al.*, 1996). During our studies the crystal structure of the bacterial homologue CorA had been reported (Lunin *et al.*, 2006;Eshaghi *et al.*, 2006;Payandeh and Pai, 2006), but the mode of ion transport remained obscure. We could finally prove that Mrs2p forms a Magnesium selective channel in the IMM with a calculated conductance of 155 pS (Schindl *et al.*, 2007). We furthermore showed that co-

expression of a homologue of Mrs2p termed Lpe10p (or Mfm1p) yielded a unique, reduced conductance in comparison to the one of Mrs2p channels (Sponder *et al.*, 2010).

6. Outlook

The progress on the electrophysiological characterization of mitochondrial channels was rather limited in the nineties with only a few labs being able to deal with the emerging technical challenges. This situation has definitely changed in the last decade: while there are not more than 100 reports on patch-clamp recordings on mitochondria indexed in pubmed in the nineties, this number has changed to more than 400 in the last decade. However, there is still a lot of work to be done. The molecular identities of most channel proteins forming the MPTP, the 107 pS channel or the mito KATP have not been defined. From that point of view the studies on the yeast Magnesium channel proteins Mrs2p and its homolog Mfm1p are of even greater relevance.

The experiences in the field of protein- and membrane reconstitution and in mitochondrial electrophysiology rendered these techniques also interesting for the characterization of other intracellular membranes. First studies by a pioneer in this field Igor Pottosin in the early nineties reported on the investigation of ion channels located in chloroplast envelope membranes (Pottosin, 1993; Heiber *et al.*, 1995) or thylakoid membranes (Pottosin and Schonknecht, 1995). Further reports described the action of a voltage-gated ion channel in the outer envelope (Hinnah *et al.*, 1997), focused on the inactivation of a 50 pS envelope anion channel necessary for import of proteins into the chloroplast (van den Wijngaard *et al.*, 1999) or characterized a fast-activating channel regulating various fluxes in the native envelope of chloroplasts (Pottosin *et al.*, 2005). The methods of choice to cope with the severe technical challenges (e.g. the maintenance of the organelle integrity) were either the reconstitution of isolated membranes into lipid vesicles – mainly based on asolectin – or measurements on whole chloroplasts. In both cases great experience as well as patience appears indispensable. That is probably the main reason for the limited number of studies in this field.

To efficiently investigate intracellular ion channels, new methods will be necessary. Assays like the one presented by Schieder and co-workers (Schieder *et al.*, 2010) using solid-matrix planar glass chips, which even enables patch-clamp recordings of lysosomes, are a step in the right direction.

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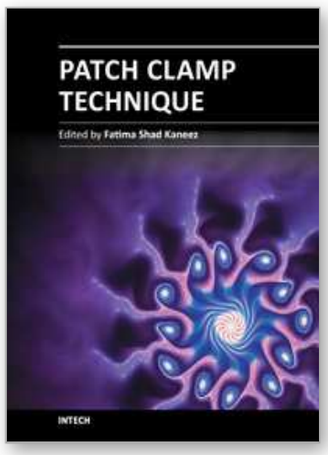

Patch Clamp Technique
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This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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