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Measuring and Evaluating the Role of ATP-Sensitive K^+ Channels in Cardiac Muscle

Eirini Kefaloyianni[1], **Li Bao**[1], **Michael J. Rindler**[2], **Miyoun Hong**[1], **Tejaskumar Patel**[1], **Eylem Taskin**[1], and **William A. Coetzee**[1,3,4]

[1]Department of Pediatrics, NYU School of Medicine, New York, NY 10016, USA

[2]Department of Cell Biology, NYU School of Medicine, New York, NY 10016, USA

[3]Department of Physiology & Neuroscience, NYU School of Medicine, New York, NY 10016, USA

[4]Department of Pharmacology, NYU School of Medicine, New York, NY 10016, USA

Abstract

Since ion channels move electrical charge during their activity, they have traditionally been studied using electrophysiological approaches. This was sometimes combined with mathematical models, for example with the description of the ionic mechanisms underlying the initiation and propagation of action potentials in the squid giant axon by Hodgkin and Huxley. The methods for studying ion channels also have strong roots in protein chemistry (limited proteolysis, the use of antibodies, etc). The advent of the molecular cloning and the identification of genes coding for specific ion channel subunits in the late 1980's introduced a multitude of new techniques with which to study ion channels and the field has been rapidly expanding ever since (e.g. antibody development against specific peptide sequences, mutagenesis, the use of gene targeting in animal models, determination of their protein structures) and new methods are still in development. This review focuses on techniques commonly employed to examine ion channel function in a electrophysiological laboratory. The focus is on the K_{ATP} channel, but many of the techniques described are also used to study other ion channels.

Introduction

ATP-sensitive (K_{ATP}) channels are expressed in diverse cell types [1]. Their activity is characteristically and principally regulated by the concentrations of intracellular nucleotides. A decrease in cytosolic ATP:ADP ratio activates the channel. Molecularly, K_{ATP} channels are composed of pore-forming subunits belonging to the inward rectifying K^+ (Kir) channel family, as well as regulatory sulfonylurea receptors (SUR), belonging to the ABC transporting family of membrane proteins [2]. The K_{ATP} channel is thought to be a hetero-octameric complex of four Kir6 subunits (Kir6.1 or Kir6.2, encoded by the KCNJ8 and KCNJ11 genes), and four SUR subunits: SUR1 (encoded by ABCC8 gene) or SUR2A/

Address for correspondence, Dr. William A. Coetzee, Department of Pediatric Cardiology, NYU School of Medicine, New York, NY 10016, USA, Tel: 1-212-263-8518, Fax: 1-212-263-5100, william.coetzee@med.nyu.edu.

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SUR2B (encoded by the ABCC9 gene) [3]. Although the cardiac ventricular K_{ATP} channel is considered to be composed of Kir6.2/SUR2A subunits (Table 1), an increased importance to other subunits (e.g. Kir6.1 and SUR1) has recently emerged [4–5]. The purpose of this treatise is not to review the physiology, molecular or disease aspects of K_{ATP} channels and the reader is referred to recent reviews on these topics [3, 6]. Instead, this review will focus on the methodology commonly used to measure K_{ATP} channel function and composition.

Electrophysiological approaches

Patch clamp recordings are most frequently used to investigate K_{ATP} channel properties and function. Microscopic recordings of unitary channel events provide much information regarding the channel, including its unitary conductance, dwell time kinetics and bursting behavior. Macroscopic K_{ATP} channel activity can also be recorded in the whole-cell recordings patch clamp configuration. Each of these recording techniques has their own advantages and disadvantages. Isolated patch experiments provide the most detailed information of the channel properties, but should be weighed against the shortcoming that data are obtained in a cell-free environment and in the absence of physiological modifiers of channel behavior. The opposite argument is true for cell-attached or whole-cell recordings. These techniques are best combined to approximate the physiological function of the channel.

Biophysical properties

The biophysical characteristics of the K_{ATP} channel are best obtained at the unitary channel level (excised or cell-attached patches). The analysis of unitary currents is described in some detail in a chapter [7] of a book that is a mandatory addition to the bookshelf of any cellular electrophysiologist, and full details will not be repeated here.

Measurement of the unitary conductance—The unitary current amplitude, defined as the current flowing when a single K_{ATP} channel opens, is most often measured with patch-clamp recordings made in the inside-out or cell-attached configurations. By plotting the unitary current as a function of the pipette potential (which can be changed by step increments or by using a slow voltage ramp protocol), one can estimate the unitary conductance. Since the K_{ATP} channel has weak inward rectifying properties, the unitary conductance is often determined as the slope conductance in a negative (and linear) range of membrane potentials. This method is advisable and is preferred over the practice of estimating the unitary conductance as the chord conductance (i.e. calculating the conductance from channel openings at a single, fixed voltage), which may not accurately account for rectifying properties of the channel (see also [8]). At any given voltage, the unitary current is most easily obtained by constructing an all-points histogram. This function is standard in most commercial patch clamp analysis software. This method is rather crude but is much more accurate than estimating the unitary current using cursors on a computer screen, which is subject to considerable variation due to the presence of noise in the recordings. The data trace (usually 10–20s of recording) should be baseline subtracted to remove baseline drift before analysis proceeds. The all-points histogram is subjected to curve fitting to a multiple Gaussian function and the unitary current is estimated as the differences between peak values. The unitary conductance of the ventricular K_{ATP} channel has been estimated to be ~78pS with high (140 mM) symmetrical K^+ concentrations [9]. The dependence of the unitary conductance (γ) on the “extracellular” K^+ concentration ($[K^+]_o$) can be approximated as:

$$\gamma = 78 + \left(\frac{[K^+]_o}{150} \right)^{0.23} \quad (1)$$

The unitary current can also be estimated from macroscopic currents obtained in the whole-cell or excised patch configurations using a technique called “variance analysis”, but this is a poor substitute for the methods described above. Fully described elsewhere [10], the basis of this method is to record both the average (I) and variance (σ^2) of the macroscopic current under conditions that vary the number of open channels. The unitary conductance is determined as the slope of a plot of the variance as a function of the mean current.

The open probability—The open probability (P_o) is estimated as the fraction of time that a channel spends in the open state, divided by the total duration of the recording. The intra-burst open probability of the ventricular K_{ATP} channel is high (>0.9) directly after patch excision and declines progressively as the channel runs down, presumably due to membrane PIP_2 depletion. The most accurate assessment of open probability (P_o) is made after event detection [7]. This is only possible if (at most) only a few channels are active in a patch. The time (t_i) spent at each current level corresponding to $i = 0, 1, 2, \ldots N$ open channels is used to calculate P_o as follows (where N is the number of channels active in the patch and T is the duration of recording):

$$P_o = \frac{\sum_{i=1}^{N} t_i}{T \cdot N} \quad (2)$$

The open probability can also be estimated from all-points histograms constructed from segments of raw data recordings where individual channel openings (usually fewer than 5–6) can be discerned. It is imperative for baseline and drift correction to be performed before constructing the histogram and fitting to a sum of multiple Gaussian distributions. From the area (A_i) of each current level of channel opening (i from 0 …. N; with N the number of apparent open channels), the P_o is calculated as:

$$N \cdot P_o = \frac{\sum_{i=0}^{N} i \cdot A_i}{\sum_{i=0}^{N} A_i} \quad (3)$$

It should be noted that open probability is often expressed as $N.P_o$ due to the uncertainty with the number of channels present in the patch that are available for opening. K_{ATP} channels are expressed at high density in cardiac myocytes (10–12 channels/patch [11] and in our experience many more, depending on the patch electrode resistance) and individual channel openings can therefore often not be discerned. Nevertheless, it is still possible to estimate $N.P_o$ in these patches by dividing the mean patch current by the unitary conductance (measured in a separate experiment or estimated from equation [1]).

Dwell time kinetics and busting behavior—Dwell time analysis should ideally be performed using recordings with only one active channel per patch. This is difficult to achieve with ventricular K_{ATP} channels because of their high expression density (3–5

channels/μm^2). The use of high resistance patch electrodes (8–12 MΩ) may help to minimize the average number of channels per patch. Data should be filtered (8-pole, low-pass, Bessel response; e.g. at 2 kHz) before being acquired to disk. The sample rate should be 5–10 times higher than the filter frequency (e.g. 20 kHz) to avoid aliasing and to satisfy the Nyquist criterion. Channel event detection is performed using a 50% threshold method [7], making sure that the detection threshold is at least three times the standard deviation of the noise (not usually a problem for a large conductance channel such as the K_{ATP} channel). Due to the limited bandwidth (time resolution) of the recording system, short openings and closings cannot be reliably detected. If left uncorrected then the brief closings within a burst are missed, resulting in an overestimate of the mean open time. A correction for these missed events must therefore performed [12–14]. The "dead time" of the recording system can be directly measured using a waveform generator [15] or estimated as 0.253/f, where f is the filter cut-off frequency [16] (e.g. ~150 μs when filtering at 2 kHz).

Event detection produces a dataset consisting of a list of channel dwell (open and closed) times. A histogram is produced for each of the closed or open states, where the abscissa depicts event duration (binned) and the ordinate the number of events within that range. Note that the first bin should start at the time equal to the "dead time" (e.g. 150 μs; see above), since there are no observations present below this value. Channel dwell time is exponential in behavior and the resulting histogram can be subjected to curve fitting to a sum of multiple exponential functions. The time constants of the exponential distributions are the mean open/closed times. The mean open time of the cardiac K_{ATP} channel is estimated to be around 2.5 ms, whereas the closed state can best be described by a sum of multiple exponential functions, with time constants ranging from around 0.5 to 3500 ms [16–19].

Bursting behavior is a typical feature of K_{ATP} channel activity. Bursts are defined as groups of rapid opening and closings, separated by longer closing events. A critical time (t_c), which separates bursts from each other, is specified [20–21], which is usually around 2.5–3.0 ms for K_{ATP} channels [17, 22–23]. The intraburst channel behavior (number of events per burst, mean burst duration) is calculated by omitting closed periods exceeding t_c, and the interburst closed times are estimated by ignoring closing events shorter than the critical time.

Channel rundown—In excised patches, the activity of K_{ATP} channels diminishes with time after patches excision; a phenomenon named "rundown" [24]. The rate of run-down is variable and is partially prevented by MgATP [24–25]. The K_{ATP} channel rundown is by prolonged exposure to high MgATP, which is thought to act via membrane PIP^2 alterations [3, 26]. In practice, run-down cannot be avoided, but can be minimized by using Mg^{2+}-containing bath solutions or intermittently exposure to high MgATP concentrations after patch excision. We typically discard data from patches exhibiting >20% rundown. In other patches, the current reduction due to rundown should be corrected during analysis (e.g. by normalizing to the current recorded in zero ATP at various times during the recording [27]).

Regulation of channel activity

Regulation by nucleotides—A hallmark feature of K_{ATP} channels is their sensitivities to cytosolic nucleotides. Their activity is blocked by free ATP, but increased by MgATP and MgADP. ATP sensitivity is measured with excised patches in the inside-out configuration. Typically, different (6–8) ATP concentrations are applied with a device that allows rapid exchanges of solutions at the cytoplasmic side of membrane. Care should be taken to avoid channel rundown (by keeping recordings as short as possible) and/or correcting for any rundown that might have occurred. Current (I) is measured and expressed as a function of

the maximal current recorded in the absence of ATP (I_o), plotted as a function of the ATP concentration and data are subjected to curve fitting to a modified Hill equation:

$$\frac{I}{I_o} = \frac{1}{1+\left(\frac{[ATP]}{IC_{50}}\right)^n} \quad (4)$$

Values for the ATP concentration that cause half-maximal inhibition (IC_{50}) and the slope of the ATP response (n) are in the range of 15–100 μM and ~1.5–2.0 for ventricular myocytes [11, 28–29]. ADP also blocks the K_{ATP} channel, but concentrations needed (IC_{50} in the mM range; [29]) exceeds cytosolic levels and this mode of channel regulation is not likely to be pathophysiologically relevant. The K_{ATP} channel activity is powerfully stimulated by MgADP. Using methods similar to that describe above, the degree of ADP stimulation is typically measured in the presence of ATP (at a concentration that produces submaximal block) [30–31]. The net effect of MgADP therefore appears as a change of the ATP-sensitivity (rightward shift of the IC_{50}).

Counting channels

Sometimes it is necessary to estimate the number of functional (open) K_{ATP} channels. A variety of techniques can be used, each with its own limitations.

Whole-cell recordings—The number of active K_{ATP} channels can be estimated from whole-cell recordings (or cell-attached patches) by measuring the K_{ATP} channel current density (current corrected for the electrically active cell surface; pA/pF). The idea is to open all available K_{ATP} channels at maximal open probability. However, this goal is not easily attained. Interventions commonly used include metabolic inhibitors (such as 2-deoxyglucose plus NaCN or dinitrophenol) [32–33], but is unclear whether these interventions activate all available K_{ATP} channels. A significant problem with this approach is that the K_{ATP} channel is very large and series resistance errors may prevent adequate patch clamp voltage control [34]. The K_{ATP} channel current may therefore be underestimated. Low resistance electrodes should be used, the access resistance should be low and every attempt should be made to electronically compensate the series resistance.

Excised patches—The K_{ATP} channel density can also be estimated from recordings made with excised membrane patches (inside-out configuration). In this case, the number of channels per patch can be estimated by Gaussian fitting of all-points amplitude histograms. In our experience, however, a typical membrane patch contains too many channels (upwards of 40) to make this method practical. By dividing the mean patch current by the unitary current, the value $N.P_o$ can be obtained, which reflects the number of channels (N) per patch (assuming a high, but constant open probability; Po). Given that the free patch area of a 2–3 MΩ pipette is estimated to be about $10\mu m^2$ [35], (or 0.01pF), the channel density can easily be calculated. The disadvantage of this approach is that P_o is never unity and that it decreases over time as the channel runs down following patch excision. The channel density may therefore be underestimated.

Noise analysis—"Variance analysis" (see earlier) can also be employed as a technique to estimate the number of channels and/or the unitary conductance [10, 36]. Under conditions when channel opening is varied, the current variance (σ^2) is plotted against the mean current (I), which results in a bell-shaped curve. The current (I) at the maximal variance is given by i.N/2, with i the unitary current and N the number of channels. The N can also be calculated when the variance becomes zero as N=I/i. Examples where non-stationary noise analysis has been used to estimate channel conductance and channel number exist for the smooth muscle

K_{ATP} channel [37–39] as well as heterologously expressed K_{ATP} channels [40–41]. A direct comparison of this technique with unitary current analysis indicated that the unitary conductance (and therefore channel number) may be seriously underestimated using this technique [42].

Expression and biochemical modulation of K_{ATP} channel subunits

The electrophysiological properties, regulation and function of K_{ATP} channels are determined largely by their molecular composition. It is therefore often necessary to identify the tissue and cellular distribution and expression levels of the individual K_{ATP} channel subunits. Moreover, channel function is strongly modulated by post-translational modifications, which makes this an important topic of investigation.

Determining mRNA distribution and expression levels

Northern blotting—K_{ATP} channel subunit mRNA expression has been quantified by Northern blot analysis, in which mRNA is extracted from the tissue/cells of interest, fractionated by size with denaturing agarose gel electrophoresis and transferred to a membrane. The membrane is then hybridized with labeled DNA probes. Northern assays have the advantage to provide information regarding the transcript size, which is particularly relevant when examining splice variants. Additionally, it provides a direct comparison of mRNA abundance in different tissues or experimental conditions. A caveat is that the RNA should be of high quality. Northern assay has been successfully used for the detection of Kir6.1, Kir6.2, SUR1 and SUR2 subunits with probes consisting of either full-length cDNA [43] or fragments of DNAs [44–46].

Reverse transcription polymerase chain reaction (RT-PCR)—RT-PCR is a sensitive method of detecting transcript expression. The mRNA is reverse transcribed and subunit and species-specific primers are used to amplify a region of the cDNA. The degree of amplification is proportional to the amount of starting material. A disadvantage of the method lies in the non-linear nature of the PCR reaction, which provides challenges when attempting to estimate comparative expression levels. This potential problem can be mitigated by keeping the number of PCR cycles low (to work within the exponential phase of amplification) or by using competitive RT-PCR for quantification purposes, for example as used to detect Kir6.2 and SUR mRNA expression levels in rat pancreatic islets [47]. An advantage includes the fact that the method allows for the detection of different size amplicons, which allows for duplex RT-PCR. It is also possible to use RT-PCR to distinguish between different splice variants, for example to distinguish between SUR2A and SUR2B subunits, which originate from a single gene (ABCC9) and differ from each other only in the C-terminal. With appropriate primer design, the presence of the alternatively spliced exon can readily be detected (for examples see Table 2). Consideration should be given on the variations of the SUR2 splicing in different species (see Figure 1). Semi-quantitative real-time RT-PCR is a more sensitive and reliable assay for detecting mRNA abundance than conventional RT-PCR [48]. Due to the fact that PCR efficiency is not perfect, primer-dependent and changing during the course of a PCR assay, care should be taken with both methods when comparing mRNA expression of different transcripts, unless a standard curve is generated for quantification purposes.

Microarray approaches—DNA microarray analysis utilizes thousands of DNA probes that are immobilized on a microchip. The mRNA of the tissue of interest is reverse transcribed to cDNA, labeled and hybridized with the probes. The probe-target hybridization is detected (usually with fluorescence) and quantified to provide information about the abundance of each target cDNA. Although this method does not specifically target K_{ATP}

channel subunits, it can be used as an initial estimate of expression levels [49–50], which should then be followed by more robust methods. A major disadvantage is that microarrays selects against transcripts with low expression levels and ion channel subunits are often undetected due to the low signal-to-noise ratio inherent with this method.

Protein expression

Western blotting—Standard Western blotting techniques are commonly used to detect K_{ATP} channel subunit protein expression. Protein extracts are size fractionated using SDS PAGE, transferred to a membrane and immunoblotted with subunit-specific antibodies. K_{ATP} abundance in most tissues is relatively low and methods should be employed that allow for enrichment. Preparations commonly used are crude membranes [51–52], plasma membranes [33, 53] or organelles [54]. Our experience is that the common practice of boiling the sample before electrophoresis should be avoided (see also [55]), most likely because of precipitation or aggregation due to the hydrophobic nature of these proteins. In our experience, the use of wet transfer with a Tris/glycine buffer supplemented with 0.01% SDS gives the best transferring results for the SUR subunits. For increasing the sensitivity of the detection, transfer of proteins on PVDF membranes is usually preferred over nitrocellulose membranes. Both semi dry and wet transfer methods can be used, although wet transfer usually facilitates the transfer of proteins larger than ~100kD, such as the SUR subunits of the K_{ATP} channel.

Success of Western blotting is highly dependent on the specificity and sensitivity of the antibodies used to detect the K_{ATP} channel subunits. Development of highly specific K_{ATP} antibodies has been a great challenge for many years and a subject of debate among investigators of the field. Up to now, many antibodies are commercially available, although many laboratories prefer antibodies that they have produced and tested. We and others found the commercial G16 and M19 antibodies (Santa Cruz) respectively to detect the Kir6.2 and SUR2A subunits [56–62]. We have also had success using anti-Kir6.2 antibodies developed by us (e.g. W62 and C62 [43, 90]), an anti-Kir6.2 antibody developed by Dr. Hon-Chi Lee (Mayo Clinic, Rochester, MN) [27, 63–64] and the BNJ2 anti-SUR2A antibody developed by Dr Jon Makielski (University of Wisconsin, Madison, WI) [65]. Testing antibodies against mouse knockout tissues has revealed interesting observations. For example, we found that native Kir6.2 protein does not only migrate at the expected size of ~37kDa, but that several other bands at higher molecular weights are observed. These bands are specific since they are absent in cardiac membrane fractions prepared from a Kir6.2 KO mouse (Figure 3). The identities of those bands remain uncharacterized, but it is possible that they represent complexes of Kir6.2 with other proteins, that are resistant to the reducing and denaturating conditions used in our laboratory. Neuromab1 (a monoclonal antibody-generating resource funded by NIH) is now developing monoclonal K_{ATP} subunits antibodies, which should assist in a better characterization of K_{ATP} channel proteins.

Binding to sulphonylureas—The levels of SUR subunits have also been estimated using sulphonylureas that are fluorescently labeled. Epifluorescence imaging of cells incubated with these substances followed by signal quantification analysis may provide information of SUR protein abundance in the cells [66] or subcellular localization of K_{ATP} channels [67]. The major advantage of this method versus Western blotting is that it is not dependent on antibody specificity. Disadvantages include the low quantification ability, the possible non K_{ATP}-specific binding of the labeled sulphonylureas and that it cannot discriminate among the different SUR subunits. Non-specific staining [68] and accumulation

[1]http://neuromab.ucdavis.edu/

inside intracellular organelles of these dyes is problematic (e.g. BODIPY® FL glibenclamide is sold as a marker of the endoplasmic reticulum).

Post-translational modifications

The SUR1 subunit of the K_{ATP} channel has been shown to be subjected to N-linked glycosylation (N10 and N1050) in the Golgi, a modification that regulates its trafficking to the membrane [69–71]. There are at least three more types of post-translational modifications of the K_{ATP} channel reported. These include Serine /Theonine phosphorylation of Kir6.1, Kir6.2, SUR1 and SUR2B [72], S-nitrosylation of SUR1 (in heterologous expression systems [73] and S-glutathionylation of Kir6.1 subunit (in heterologous expression system and vascular smooth muscle cells [74]). Standard biochemical methods have been used for the detection of those modifications since K_{ATP} channel-specific tools are not yet available (such as phospho-specific antibodies).

Tissue distribution and subcellular localization

mRNA tissue distribution—mRNAs encoding Kir6.x and SURx isoforms have been detected in the heart using *in situ* hybridization techniques, including autoradiography on whole fixed and paraffin embedded mouse embryos [75] and riboprobe methodology on frozen sections of fixed tissues [76]. The latter method, however, may not be as sensitive, as it failed to show expression of SUR2B in myocardium, in contrast to the autoradiography study, Northern blot analyses, and immunolocalization studies.

Subcellular localization of proteins

Immunofluorescence and light microscopy: Native K_{ATP} channel subunits have been localized in dissociated cardiomyocytes or cultured neonatal myocytes by fluorescence and by confocal microscopy [53, 62, 77–81]. Prior to immunostaining using standard techniques, cells were fixed in paraformaldehyde and permeabilized with methanol or Triton X-100, depending on the properties of the antibodies used. Alternatively, cells were fixed/ permeabilized in 1:1 methanol/acetone [77]. The major consideration in these and all other localization studies is the specificity and quality of the antibodies. K_{ATP} channels have also been localized in sections of heart tissue. Cryosections prepared from paraformaldehyde-fixed cardiac tissue have been stained for confocal microscopy using antibodies to Kir6.1 and Kir6.2 [61, 80]. Conventional horseradish peroxidase (HRP) staining methods have been applied to cryosections (anti-SUR antibodies) and paraffin sections (anti-Kir6.x antibodies) [78, 82]. One caveat is the poor resolution of HRP staining at the light microscopic level.

Immunoelectron microscopy: Cryosections stained with HRP-coupled secondary antibodies for light microscopy have also been post-fixed with osmium, embedded in resin, and sectioned for visualization at the EM level [61, 82]. In these studies, Kir6.x and SUR2x were found in the ER, mitochondria, and sarcolemma of cardiomyocytes. The major problem with this method is that HRP is not particularly quantitative at the EM level and can diffuse. A more quantitative method using ultrathin frozen sections of paraformaldehyde-fixed cardiac tissue has also been employed to localize K_{ATP} channels. The methods for preparing ultrathin frozen sections and immunostaining using colloidal gold coupled secondary antibodies are essentially the same as those used for other tissues [83]. The results of channel localization have been controversial as the sarcolemmal Kir6.1 and Kir6.2 subunits were reported also to be present in mitochondria in one study [61], while another study concluded that the localization of Kir6.1 to mitochondria was due to antibody cross-reactivity with mitochondrial proteins [80].

Modulation of K_{ATP} channel expression by siRNA

In many studies it is important to modulate the expression levels of the K_{ATP} channel subunits. The method of siRNA has been successfully employed to eliminate the expression of the K_{ATP} proteins in heart cells. This includes the use of small RNA molecules (siRNA oligonucleotides) that are designed to specifically bind the mRNA of the subunit of interest, resulting in the reduction of the translation of this transcript. The advantage of the direct down-regulation of the protein is that it may provide evidence for the functional roles of the protein under different experimental conditions. For example, siRNA oligonucleotides targeting Kir6.2 and Kir6.1 have been used to demonstrate a role for the K_{ATP} channel in hypoxic preconditioning in H9c2 cells [54] and silencing of SUR2 in rat neonatal cardiomyocytes has been used to demonstrate the role of K_{ATP} channel in the expression of peroxisome proliferator-activated receptor γ coactivator (PGC)-1^{α} [84]. Disadvantages include the possible non specific action of the siRNA oligonucleotides utilized and that the method can be applied only in *in vitro* models (cells in culture).

Trafficking of K_{ATP} channel subunits

Cell Fractionation

Cell Fractionation has been used to study the subcellular localization and trafficking of K_{ATP} channels both on ventricular tissue [33, 85] and on isolated cardiac myocytes [86]. Differential centrifugation or density gradient methods were used to separate plasma membranes from endosomes and mitochondria. The differential centrifugation method is based on the fact that mitochondria sediment at relatively low (4–10,000*g*) rate centrifugal force (RCF) while plasma membranes are recovered at intermediate (20–30,000*g*) and endosomes and microsomes at higher RCF [78, 85–87]. Preincubation of cardiac tissue in high salt buffers, which dissociate actin filaments and remove some peripheral membrane proteins, prior to homogenization improves separation [87]. Bao et al [33] combined high salt preincubation with an Optiprep step gradient protocol to separate sarcolemma and endosomal fractions [88]. Sucrose step gradients have also been used to prepare a sarcolemma membrane fraction containing K_{ATP} channels [62]. The purity of cellular organelles prepared from cardiac tissue in which actin/myosin filaments predominate has been a major challenge and the source of controversy in the field, especially with regard to whether sarcolemmal K_{ATP} channel subunits are present in mitochondria [see [89]].

Caveolae are cholesterol and sphingolipid-enriched invaginations of the plasma membrane involved in endocytic and signal transduction events. Because of their light density, they can be separated from most other cellular membranes on sucrose step gradients after removal of peripheral membrane proteins with sodium carbonate [53]. K_{ATP} channel subunits were shown to fractionate with caveolar membranes obtained from cardiomyocytes and to interact with caveolar component proteins.

Cell Surface Labeling Techniques

Because antibodies against extracellular epitopes of K_{ATP} channel subunits have not been available, detection of native channels at the cell surface using immunological techniques has not been feasible. However, it has been possible to label K_{ATP} channels and follow their internalization. Epitope tags or bungarotoxin binding sequences have been introduced into the extracellular loop of Kir6 or at the amino terminus or an internal extracellular sequence of SUR using molecular biological techniques that are beyond the scope of this review. The modified constructs are incorporated into expression vectors or recombinant adenovirus and the proteins expressed exogenously in cardiac myocytes and other cell types [90–92]. Quantitation can then be performed using ELISA assay [90] or the antibodies visualized by confocal microscopy [33, 90, 93–94]. Internalization and recycling of channels can also be

observed by similar techniques [92, 95]. Biotinylation is another technique that has been used to quantify cell surface K_{ATP} channels, albeit in cells expressing high levels of the channels after transfection [95].

Pulse-chase labeling

Pulse-chase radiolabeling can be used to monitor the rate of transport of SUR subunits out of the endoplasmic reticulum (ER). Briefly, cells are pre-incubated in methionine- and cysteine-free medium (30 min – 2 h) and labeled in the same medium containing [35]S-met and cys (e.g., Translabel from ICN) for 30 min - 1 h. After washing, the cells are incubated in a medium supplemented with unlabeled met and cys (5 mM) for various periods of time, after which cells are solubilized in Tris EDTA buffer with detergents (e.g., 1% Triton X-100, see co-IP section). SUR subunits are visualized on a phosphorimager after immunoprecipitation and SDS-PAGE (8% polyacrylamide or a gradient gel). Because they are N-glycosylated, SUR1 subunits migrate as 2 bands on the gels – an initial band appearing after the pulse and representing the core glycosylated ER form as well as a second, slightly larger band appearing later and corresponding to the terminally glycosylated form that has traversed the Golgi apparatus. Pulse-chase labeling has been informative in characterizing the roles of hsp90 and syntaxin-1A in SUR1 trafficking out of the ER [96–97]. A similar protocol has been used to monitor rates of turnover of KATP channel subunits (e.g. [98]).

K_{ATP} channel complexes

It is getting increasingly acknowledged that native K_{ATP} channels consist, apart from the channel's subunits Kir6.x and SURx, of a macromolecular protein complex that regulates their function. K_{ATP} interacting proteins can be identified using large scale proteomic assays (such as bacteria/yeast two hybrid systems or mass spectrometry) as well as smaller scale assays (such as co-immunoprecipitation).

Co-immunoprecipitation

This method includes immunoprecipitation (IP) of the subunit of interest under conditions which retain the interaction of the proteins, followed by detection of putative interacting proteins in the immunoprecipitate. The detection method often includes Western blotting, if the possibly interacting protein is known and antibodies against it are available [51], as well as mass spectrometry methods if the interacting protein is not known [99–100]. It is important that appropriate solubilization of the proteins is performed. The method used (detergents, sonication, etc.) should be carefully selected in order to effectively solubilize the proteins of interest without disturbing the possible interactions. For the cardiac K_{ATP} channel, solubilization in 1% Triton X100 has been widely used (for example [51, 53, 100]. For the same reason, incubations in high ionic strength buffers should not be included during the sample preparation and IP (a broadly used salt concentration for such experiments is approximately 150 mM, [51, 53, 100]. The success of this method is largely dependent on antibody quality (see also Western blot section). For the verification of the interaction, the use of multiple IP antibodies is suggested as well as use of the reciprocal IP.

Proteomics

Yeast/bacterial two hybrid systems—Two hybrid systems are based on transcription factors that consist of an activating and a DNA binding domain that can function only when they are in close proximity. Plasmids are produced that contain either the protein of interest (bait) fused with one part of the transcription factor or a group of target proteins (pray, often a large cDNA library) fused with the second part of the transcription factor. The bait and pray plasmids are simultaneously introduced into the yeast/bacteria and an interaction

between proteins of the bait and pray will result in activation of the transcription factor and subsequent transcription of reporter genes. A bacteria hybrid system has been used to identify interacting proteins of the pore forming subunit of the cardiac K_{ATP} channel, Kir6.2 using a truncated version of the protein (residues 169–354) as a bait [51]. Moreover, a yeast two-hybrid system optimized for membrane protein interactions (MbYTH) has been used to verify the interaction of Kir6.2 with GAPDH [101]. Partial rat SUR1 (residues 598–1003) has been also used in screening a pancreatic cell cDNA library [102]. One of the advantages of these methods is that they are not antibody-dependent. However, controlling for false positive and negative reactions might be a point of criticism and the results are usually confirmed by other methods (such as co-IPs).

Mass spectrometry—Mass spectrometry methods are increasingly used in identification of protein interactions because of their sensitivity and the ability to provide quantitative results. When they are used in combination with co-IP assays they may lead to identification of unknown interacting partners of a protein. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis has been used to identify creatine kinase and the muscle form of LDH as interacting proteins cardiac K_{ATP} channel immunoprecipitates [99–100]. Recently, co-IP followed by tandem mass spectrometry was used to identify putative interacting proteins of the K_{ATP} channel in the rat ventricle [64]. Moreover, linear ion trap quadrupole-Orbitrap mass spectrometry was used in combination with two dimensional gel electrophoresis in identifying differentially expressed proteins in K_{ATP} channel deficient hearts under control or disease conditions [103–105].

Genetic approaches

Targeting K_{ATP} channels at the genetic level, either for overexpression or ablation, has been a powerful approach in studying the roles of diverse K_{ATP} channels in a variety of tissues. A full review of data obtained from these genetic approaches is beyond the scope of this paper and the reader is referred to other reviews for this information [106–107]. Two main approaches have been used to date: the first relies on the transgenic overexpression wild-type or mutant K_{ATP} channel subunits, and the second relies on gene targeting.

Transgenic mice

A transgenic animal is one in which a foreign gene (often a cDNA under the control of a tissue-specific promoter) has been deliberately inserted into its genome. The genomic insertion of the transgene is a random process. For practical reasons (well-defined genetics, small size, short breeding cycle and low housing costs in comparison to larger vertebrates) mice have become the principal species used for transgensis in biomedical research. Other advantages of transgensis are the lower cost and shorter time to produce offspring compared to other genetic approaches. Disadvantages include the fact that transgene insertion into the genome is random and control over expression is not possible. High expression levels of the transgene and the randomness of insertion may also disrupt the expression and/or function of other genes. Despite these limitations, transgensis has been successfully used to further our understanding of K_{ATP} channels in the intact animal (Table 3). For example, pancreatic expression of a pore-mutant, dominant negative or wild-type Kir6.2 subunits [108–109] was shown to affect pancreatic β-cell function. Cardiac-specific overexpression of dominant negative Kir6.x subunits was shown to reduce the tolerance of mice to exercise-induced stress [59]. Cardiac overexpression of ATP-insensitive or wild-type K_{ATP} channel subunits lead to diverse and complex phenotypical alterations in K_{ATP} channel activity and cardiovascular function [110–111]. We developed transgenic mice that allow tissue-specific expression of a pore-mutant Kir6 subunit (using the Cre-lox technology). These mice have been used to demonstrate that specific endothelial expression results in hypertension and

abnormalities in endothelin-1 release [112] and that skeletal muscle-specific expression alters energetics [113].

Gene targeting

Gene targeting is an approach in which a specific gene is being altered by the process of homologous recombination. Gene targeting is almost exclusively performed in mice. The targeting can take the form of changing the entire gene or just a few key exons. These regions can be deleted (knockout) or replaced with another genetic element (knock-in). Examples of knock-ins include the replacement of a gene with a marker protein (useful for examining the tissue expression of a protein of interest) or with a mutant form of the same protein (useful for determining in-vivo effects of specific human mutations). A major advantage of gene targeting is that a specific gene is deleted, which should have less impact to dysregulate gene expression. For knock-ins, another advantage is that expression is under the control of endogenous promoters/enhancers, which allows for natural control of gene expression. A disadvantage is the high cost associated with gene targeting and the time it takes to generate these mouse models. For K_{ATP} channel subunits, the only published examples of gene targeting to date include the generation of knockout mice. Knockout mouse models have been developed in which expression of each of these K_{ATP} channel subunits has been eliminated. The availability of these animal models has done much to advance our knowledge of the functions of K_{ATP} channels in the *in-vivo* setting (Table 3). The KCNJ8 gene (Kir6.1) can be found on mouse chromosome 6 at location 142,522,146–142,528,581 and contains 3 exons. The coding region of Kir6.1 is contained in exons 2 and 3. In the Kir6.1 null mouse, the strategy was to delete coding region of exon 3 [114]. Phenotypically, these mice develop vascular problems (they lack vascular K_{ATP} channels and develop coronary artery spasms that resemble Prinzmetal (or variant) angina in humans. The KCNJ11 gene (Kir6.2) is found on mouse chromosome 25 at location 19,105,218–19,106,363. It has a single exon containing the entire coding region, which has been targeted for deletion [115]. These knockout mice largely lack the expected hyperglycemic phenotype, but have been used to demonstrate that Kir6.2 is a key regulator of glucose- and sulfonylurea-induced insulin secretion. These mice have also been used (amongst others) to demonstrate a role for sarcolemmal K_{ATP} channels in ischemic preconditioning [116] and that K_{ATP} channels protect against deleterious effects of stress [117]. The SUR1 subunit contains 17 transmembrane segments. The ABCC8 gene, which codes for SUR1, is found on mouse chromosome 25 at location 19,072,876–19,091,318. It is comprised of multiple exons and several SUR1 splice variants have been described. Two groups have produced SUR1-null mice by deleting exon 1, which contains promoter regions and the starting methionine [118], or exon 2 [119]. Although neither mouse model displayed the expected severe dysregulation of insulin release, they have been useful in helping us to understand the fine tuning of insulin release and other aspects of SUR1 function elsewhere in the body. For example, the SUR1-null mice unexpectedly are protected from cardiac ischemia [120] and they have been helpful in the identification of a novel subtype of K_{ATP} channels in mouse atria [52]. The SUR2 subunit is coded by the ABCC9 gene, which is found on mouse chromosome 6 at location 142,546,356–142,659,537. The mouse ABCC9 gene has 40 exons (who of which are untranslated). The two major splice variants (SUR2A and SUR2B) differ from each other in alternative use of exons 39 and 40. In the SUR2 null mice, two exons were targeted that code for regions in the first of the two intracellular nucleotide binding folds [121], which are involved in ATP/ADP binding. Similar to the Kir6.1-nulls, these mice are hypertensive and display coronary vasospasms [122]. These mice are paradoxically protected against ischemia [123], but the data interpretation is complicated by the presence of SUR2 isoforms that are still expressed in these mice [124].

Future directions

Although powerful, existing genetic mouse models have not been able to fully address questions related to the function of K_{ATP} channels. If anything, emerging data using these models have served to highlight that we do not fully understand the complexity of K_{ATP} channel function, and in particular, the important interactions that may occur between channel subtypes in different tissues. Several groups are currently working on strategies to conditionally knock out K_{ATP} channel subunits in a tissue-specific manner. Temporal control of gene deletion can also be useful in some experimental designs, but may be less of an issue since lethality has not been reported to be an issue with the existing knockout mice. Finally, it may be of interest to produce disease-causing knock-in mouse models (e.g. the E23K Kir6.2 variant) to examine the role of polymorphisms and mutations in disease [6].

Computer modeling

A full understanding of ion channel function is often reflected in the maturity of mathematical models describing their function and in the accuracy by which the computational model can predict known and unknown (yet to be tested) behaviors. Some channels are well characterized and excellent models exist; for example, modeling the L-type Ca^{2+} channel and its regulation by signalling cascades or Ca^{2+} movements within restricted spaces within a cardiac myocyte. The opening behavior of K_{ATP} channels has also been subject to numerical modeling. At the single channel level, K_{ATP} channels open in bursts. The closing time between bursts can be described by the summation of several closed states. This complicated state behavior of K_{ATP} channel channels is best simulated using Markovian models. The reader is referred to an excellent recent review, which provides a comparative analysis, strengths and weaknesses of several state-dependent numerical models of the K_{ATP} channel to describe its ATP-dependent and ATP-independent opening kinetics [125]. Existing models are classified into “independent” models, where at rest each of the four K_{ATP} channel pore-forming subunits independently alternate between the active/inactive states and the channel is closed when one or more of the channel subunits enter the inactive state [126–127]. The independent gating of subunits has been questioned [128] and the possibility exists that pore-forming subunits may interact with each other during the interburst open/closed transitions. This possibility is incorporated in the “concerted” models, which assume that all four Kir6.x subunits undergo a simultaneous state transition. These models provide a better description of some K_{ATP} channel characterizes, including inhibition of the channel by ATP [125]. Other aspects of K_{ATP} channel gating regulation are less well described numerically. For example, the biphasic effects of MgADP, which promotes K_{ATP} channel opening at low concentrations but inhibits the channel at higher concentrations, has been modeled for the cardiac K_{ATP} channel [129], but has not been widely adopted. Other aspects of K_{ATP} channel function, including changes in permeation (e.g. channel rectification induced by polyamines and monovalent cations), regulation by pH and signalling cascades) have largely not been implemented and await future efforts. Numerical models that do not attempt to simulate the K_{ATP} channel state dependence also exist [130]. The model of Ferrero et al [130] accounts for the regulation of the K_{ATP} channel by several intracellular factors ATP/ADP ratio, pH, Mg^{2+}, and Na^{+}) and is therefore often used in computational simulations of the metabolically impaired cardiac myocyte.

Highlights

- We review methods for measuring K_{ATP} channel in cardiac muscle
- Methods covered include electrophysiological approaches and mRNA and protein expression

Other methods discussed include trafficking, complex formation and genetic approaches

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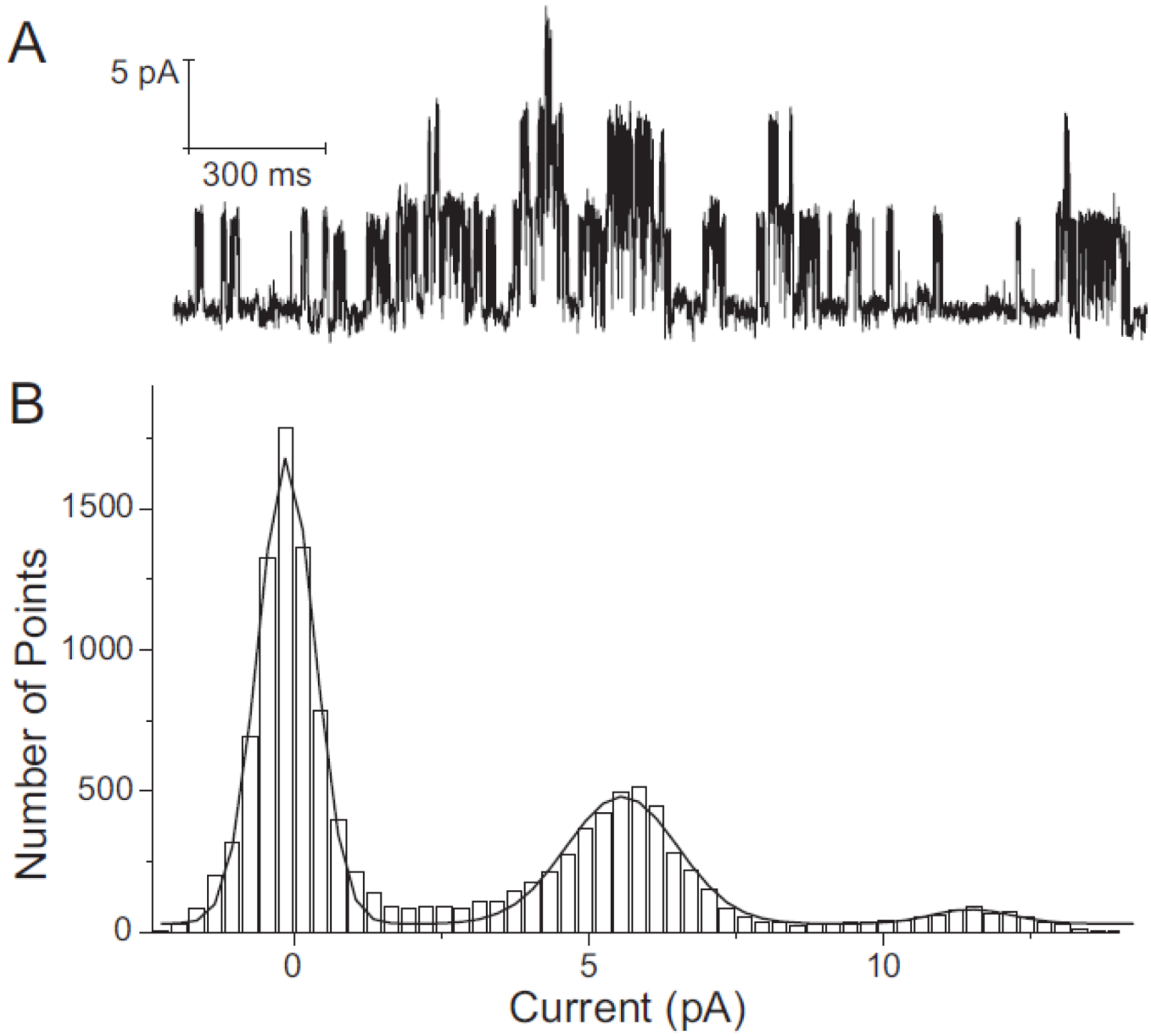

Figure 1.

Typical all-points amplitude histogram of the K_{ATP} channel. A: Recording of K_{ATP} channel activity in an inside-out membrane patch from a rat ventricular myocyte using symmetrically high (140mM) K^+ concentrations in the pipette and bath solutions. The recording, which contains three active K_{ATP} channels, was made at room temperature at a membrane potential of −80 mV. Inward currents are shown as upward deflections. B: An all-points histogram of the current segment shown in Panel A, with bin width set at 0.3 pA. The solid line is a sum of three Gaussian functions fitted to the experimental data. The distance between peaks is ~5.8 pA, which represents the unitary amplitude of the K_{ATP} channel.

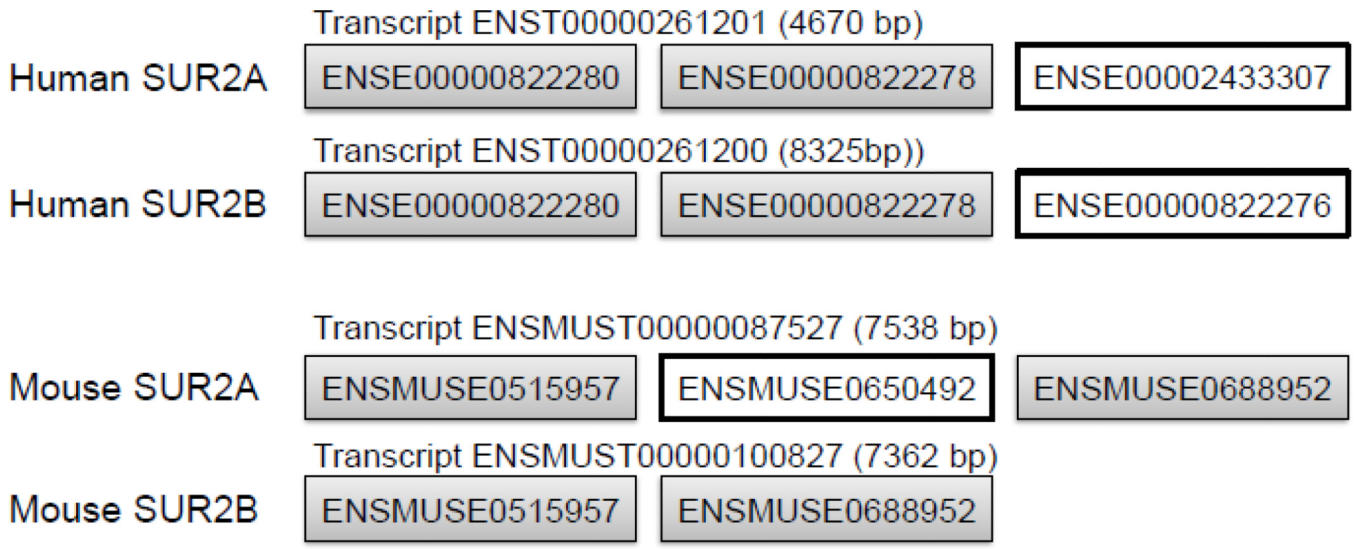

Figure 2.

Species specific considerations in separating the SUR2A and SUR2B transcripts by RT-PCR. The C-termini of the mature mRNA of SUR2A and SUR2B of human and mouse origin are shown (ENSEMBL exon numbering). In order to separate between the two transcripts, different sets of primers need to be used in the case of mRNA of human origin, with the reverse primers designed in the last exon. For the mouse, the same set of primers can discriminate between the two transcripts if the two primers are designed across the alternatively spliced exon. The rat Abcc9 gene has a similar exonic organization as the mouse (not shown).

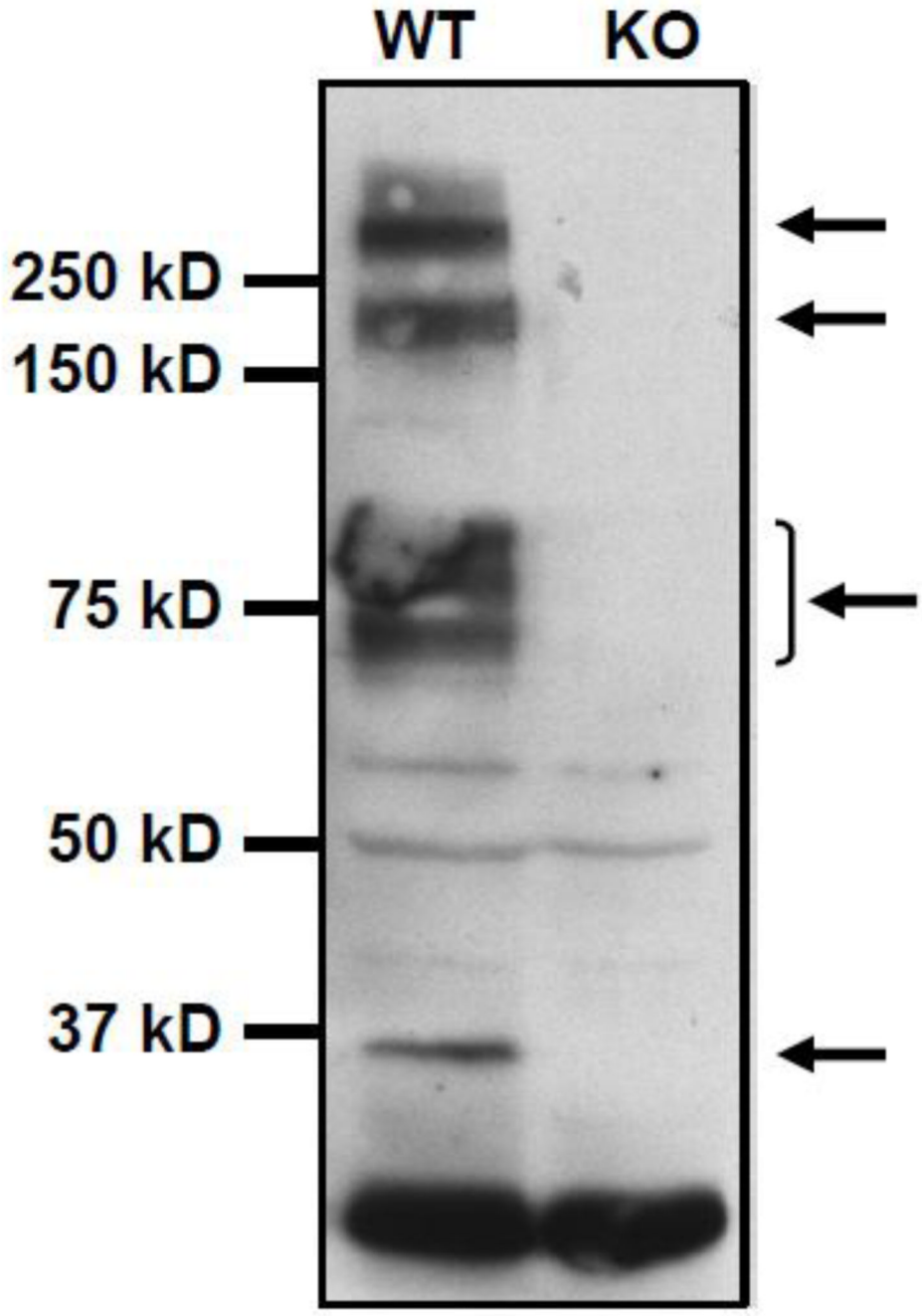

Figure 3.

Characterization of mouse cardiac Kir6.2 subunits by Western blotting. Heart membrane extracts from wild type (WT) and knock out (KO) mice were prepared and the expression of Kir6.2 was examined with SDS-PAGE using the C62 antibody developed by us against the peptide Ac-CVAKAKPKFSISPDSLS-OH (corresponding to the C-terminal 15 amino acids of human, mouse, rat, dog, bovine and horse Kir6.2. Note that samples were not heated before loading (see text). A specific band (i.e. present in the sample from the WT but not the KO mouse) was detected approximately at the level of 37 kD marker (Precision Plus Protein Kaleidoscope Standards, #161-0375, BioRad). This is a band commonly used for the study of this protein. However it is notable that higher order bands of high intensity were also

present (arrows). These bands are specific since they were absent in cardiac membranes prepared from Kir6.2 knockout mice.

Table 1

Subunit composition of K_{ATP} channel subunits in various cell types of the cardiovascular system.

Cell type	Pore forming subunits	Auxiliary subunits	References
Ventricular	Kir6.2	SUR2A	[18]
Atrial	Kir6.2	SUR1	[52, 131]
Purkinje	Kir6.1 / Kir6.2	SUR2B	[27]
Smooth muscle	Kir6.1	SUR2B	[45–46]
Endothelial	Kir6.1 / Kir6.2	SUR2B	[57, 132]

Table 2

Primer sequences which have been used to discriminate between SUR2A and SUR2B transcripts by conventional RT-PCR

Forward Primer	Reverse Primer	Product size (base pairs)	Ref.
5’-CCAGCTGAAGAATATGGTCAAA-3’ mouse exon 37	5’-CAGGTCTGCAGTCAGAATGG-3’ mouse exon 40	447 for SUR2A 271 for SUR2B	[27]
5’-ATCATGGATGAAGCCACTGC-3’ mouse exon 38	5’-AACGAGGCAAACACTCCATC-3’ mouse exon 40	357 for SUR2A 181 for SUR2B	[58]
5’-ATATGGTCAAATCTCTACCTGGAGG-3’ human exon 35	5’-GTTGGTCATCACCAAAGTGGAAAAG-3’ human exon 38	394 for SUR2A	[133]
5’-ATATGGTCAAATCTCTACCTGGAGG-3’ human exon 35	5’-CATGTCTGCGCGAACAAAAGAAGC-3’ human exon 38	397 for SUR2B	[133]

Table 3

Genetically modified mice in the study of the K_{ATP} channel.

Transgenesis				
Subunit targeted	*Type of modification*	*Cell type targeted*	*Functions modified/studied*	*References*
Kir6.2	Pore mutant	Pancreatic	Pancreatic β-cell function	[108–109]
Kir6.1, Kir6.2	Pore mutant	Cardiac	Exercise stress, K_{ATP} activity	[59]
Kir6.2	ATP insensitive	Cardiac	Cardiovascular function	[110]
SUR1, SUR2A	Overexpression	Cardiac	Cardiovascular function	[111]
Kir6	Pore mutant	Endothelial	Hypertension, endothelial endothelin-1 release	[112]
Kir6	Pore mutant	Smooth muscle	Energetics	[113]
Gene targeting				
Subunit targeted	*Total number of exons*	*Exon(s) targeted*	*Functions modified/studied*	*References*
Kir6.1	3	3rd	Vascular	[114]
Kir6.2	1	1st	Stress, ischemic preconditioning, insulin release	[115–117]
SUR1	39	1st	Insulin release	[118]
SUR1	39	2nd	Insulin release, cardiac ischemia, atrial K_{ATP}	[52, 119–120]
SUR2	41	12th to 16th	Vascular, cardiac ischemia	[65, 121–123]

Exons are numbered according to the cited references.