Differences in the mechanism of metabolic regulation of ATP-sensitive K\(^+\) channels containing Kir6.1 and Kir6.2 subunits

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Aims ATP sensitive K\(^+\) channels (K\(_{\text{ATP}}\)) sense adenine nucleotide concentrations and thus couple the metabolic state of the cell to membrane potential. The hetero-octameric complex of a sulphonylurea receptor (SUR2B) and an inwardly rectifying K\(^+\) channel (Kir6.1) and the corresponding native channel in smooth muscle are relatively insensitive to variations in intracellular ATP. Activation of these channels in blood vessels during hypoxia/ischaemia is thought to be mediated via hormonal regulation such as cellular adenosine release or the release of mediators from the endothelium. In contrast, intracellular ATP prominently inhibits Kir6.2 containing complexes, such as those present in cardiac myocytes. Thus, we investigated differences in the mechanism of metabolic regulation of Kir6.1 and Kir6.2 containing K\(_{\text{ATP}}\) channels.

Methods and results We have heterologously expressed K\(_{\text{ATP}}\) channel subunits in HEK293 and CHO cells and studied their function using \(^{86}\)Rb efflux and patch clamping. We show that rodent Kir6.1/SUR2B has direct intrinsic metabolic sensitivity independent of any regulation by protein kinase A. In contrast to Kir6.2 containing complexes, this was not endowed by the ATP sensitivity of the pore forming subunit but was instead a property of the SUR2B subunit. Mutagenesis of key residues within the nucleotide-binding domains (NBD) implicated both domains in governing the metabolic sensitivity.

Conclusion Kir6.1/SUR2B has intrinsic sensitivity to metabolism endowed by the likely processing of adenine nucleotides at the NBD of SUR2B.

KEYWORDS Experimental; Vasculature; Cellular; Electrophysiology; Ion channels; Ion transport; K-ATP channel; K-channel; Smooth muscle

1. Introduction

Cells and tissues can respond in variety of ways to hypoxia and ischaemia and in systemic vascular beds, vasodilatation is a key in matching demand and supply. Historically, a major hypothesis is that an increase in adenosine in the cellular interstitium, generated by ecto 5'-nucleotidase and acting on vascular smooth muscle (VSM), is responsible for hypoxic vascular reactivity and exercise hyperaemia particularly in the coronary circulation. However, other pathways may well be involved including the endothelium with adenosine-dependent and -independent nitric oxide and prostacyclin release. A number of studies indicate that much of the vascular response is sensitive to glibenclamide or other ATP-sensitive K\(^+\) channel (K\(_{\text{ATP}}\)) inhibitors and adenosine may act via a kinase mediated, particularly cAMP-dependent protein kinase (PKA), regulation of smooth muscle K\(_{\text{ATP}}\) channels.

K\(_{\text{ATP}}\) channels in tissues other than smooth muscle can directly sense changes in intracellular adenine nucleotide concentration. For example, in pancreatic ß-cells, this property is key to their central role in stimulus–secretion coupling. At the molecular level, the K\(_{\text{ATP}}\) channel is a hetero-octameric complex comprising an inwardly rectifying K\(^+\) channel subunit, either Kir6.1 or Kir6.2, and a regulatory sulphonylurea receptor (SUR) subunit. In cardiac, pancreatic, and other tissues, there is a ‘classical K\(_{\text{ATP}}\)’ channel with a 70–80 pS single-channel conductance that is inhibited by ATP, stimulated by MgADP, and that has activity in the absence of dinucleotides. Such channels seem to be constituted of Kir6.2 together with a SUR subunit. The molecular basis of ATP sensitivity in Kir6.2 containing complexes has been studied in detail, has led to the identification of key residues within the pore forming subunit interacting with ATP and also a structural model of that binding site. SUR is a member of the ATP-binding cassette (ABC) superfamily of transporter and contains two nucleotide-binding domains (NBD) that can bind and hydrolyse adenine nucleotides. In other ABC transporters, mutations within the

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Walker A motifs of the NBD were shown to abolish ATP binding and/or hydrolysis\(^{12}\) and in SUR1 these mutations (or analogous ones) prevent activation by Mg-nucleotides and ATP binding and/or hydrolysis.\(^{13,14}\) Finally, co-expression of Kir6.x and SUR is necessary to generate significant plasmalemmal currents, and both subunits are required for cell-surface expression of the channel. Kir6.x and SUR contain Arg–Lys–Arg motifs that act as a quality control mechanism, ensuring surface expression of only properly assembled octameric proteins.\(^{15}\)

The \(K_{\text{ATP}}\) current in smooth muscle is less sensitive to changes in intracellular ATP and the opening of these channels is absolutely dependent on the presence of nucleotide diphosphates.\(^{16}\) These currents can also be activated characteristically through a PKA dependent mechanism.\(^{18}\) A variety of studies indicate that the vascular \(K_{\text{ATP}}\) is constituted at the molecular level by Kir6.1/SUR2B.\(^{17,18}\) Furthermore, the channel activity of the cloned Kir6.1/SUR2B channel complex is also absolutely dependent on the presence of nucleotide diphosphates and is not prominently inhibited by ATP.\(^{19,20}\) Given these issues, we have investigated whether Kir6.1/SUR2B channels are directly regulated through metabolic perturbation and if so what molecular mechanisms might account for this behaviour.

### 2. Materials and methods

#### 2.1 Molecular biology

Point mutations were introduced into SUR2B with site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA, USA) according to the instructions of the manufacturer. N- and C-terminal truncations of Kir6.1 were made via conventional PCR. A plasmid encoding Kir6.1 with an HA epitope introduced into the extracellular M1 and H5 domains (Kir6.1-eHA) was kindly provided by Dr. LY Jan, and sub-cloned into the mammalian expression vector pcDNA3.1/Zeo.

#### 2.2 Cell culture

Expression was carried out in HEK293 cells or CHO-K1 cells, cultured as described previously.\(^{21,22}\) HEK293 cells were generally our assay system of choice, but surface staining assays have been shown to work better in CHO-K1 cells.\(^{23}\) The stable lines were used as before (HEK293 cells containing either Kir6.1 with SUR2B or Kir6.2 with SUR2B).\(^{24}\) Transient transfections were performed using lipofectamine with enhanced green fluorescent protein as a marker for transfected cells.

#### 2.3 \(^{86}\)Rubidium flux

\(^{86}\)Rb\(^{+}\) flux was used as a congener for K\(^{+}\) transport through \(K_{\text{ATP}}\) channels. Monolayers of HEK293 cells were grown and transfected in six-well plates. Twenty-four hours after transfection, cells were incubated overnight at 37°C in MEM containing 0.037 MBq/mL \(^{86}\)RbCl. The medium was aspirated, and cells washed three times with assay medium (in mmol/L: 10 HEPES, 10 glucose, 130 NaCl, 7 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\) adjusted to pH 7.4), and incubated for 15 min with 2 mL of assay medium with or without channel stimulants and/or inhibitors at room temperature. Cells were pre-incubated for 15 min with AMPK activators/inhibitors, PKA inhibitors, and adenosine receptor antagonists prior to addition of metabolic inhibitors. Following incubation, the supernatant was aspirated into vials for counting. Radioactivity in the supernatant and lysate was counted and efflux was expressed as a percentage relative to the total amount of radioactivity initially loaded into the cells (the sum of the radioactivity present in the supernatant and lysate).

#### 2.4 Electrophysiology

Membrane currents were studied with the perforated patch-clamp technique using an Axopatch 200B amplifier (Axon Instruments), a Digidata 1200B board for analogue to digital conversion, and analysed using pClamp software (version 6.0; Axon Instruments). Series resistance was compensated to ~70% using the amplifier circuitry. Amphoteriicin B from streptomycetes species (Sigma-Aldrich) was used to obtain the whole-cell perforated patch configuration. The bath solution contained (in mmol/L) 130 NaCl, 5.4 KCl, 10 HEPES, 2.5 CaCl\(_2\), and 1 MgCl\(_2\). The pipette solution (pH 7.4) contained the following (in mmol/L): 135 K (107 KCl and KOH to pH 7.4), 5 NaCl, 10 EGTA, 10 HEPES, 1 CaCl\(_2\), and 2 MgCl\(_2\). Pipettes were pre-dipped in regular pipette solution for around 10 s, then back-filled with amphoteriicin B-containing pipette solution (120 μg/mL). Perforation was complete within 10 min and all recordings were made at room temperature. Drugs were applied using a gravity-driven perfusion system.

#### 2.5 Immunohistochemistry

Surface staining was performed to localize HA-tagged Kir6.1/N\(_{\text{N}}\)/ΔC truncation mutants in CHO-K1 cells and was carried out as previously described.\(^{25}\) Slides were viewed using a computer-based image analysis system (OPENLAB 3.1; Improvision, Cambridge, UK), coupled to a Zeiss Axiosvert 100 M microscope equipped with epi-fluorescence illumination.

#### 2.6 Data analysis

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used to calculate statistical significance. Data presentation and statistical analysis were performed using Origin 6.0 (Microcal) or Prism v3.0 (GraphPad).

#### 2.7 Reagents

Compounds were from VWR international with the exception of forskolin (Calbiochem), glibenclamide, pinacidil (Sigma), levcromakalim, PNU-37883A (Tocris Cookson), H-89, KT5720 (Santa Cruz Biotechnology), SCH58261, and quinoxaline (Sigma).
Mitochondrial electron transport chain (with NaCN) and inhibition of glycolysis (with 2-DG) were necessary to induce KATP channel opening (Figure 1C) in the experimental time-frame examined.

We subsequently measured whole-cell current responses using the perforated patch clamp technique to confirm the response of Kir6.1/SUR2B (Figure 2A) and Kir6.2/SUR2B to metabolic inhibition (Figure 2B). Currents began to increase.

**Figure 1** The K<sub>ATP</sub> channels Kir6.1/SUR2B and Kir6.2/SUR2B are metabolically sensitive. (A) The time course of ⁸⁶Rb<sup>+</sup> efflux in HEK293 cells that stably and transiently express channel subunits. Lev, levcromakalim; glib, glibenclamide. (B) A summary of the efflux observed after 15 min of treatment with the agents as indicated. (C) Both metabolic inhibitors are required to induce the channel opening observed in this setting. All data are shown as ± SEM (n = 9) and the control represents an equivalent concentration of DMSO diluted in buffer. ***P < 0.001 compared to control, (ns) P > 0.05 compared to control.

**Figure 2** Kir6.1/SUR2B stable line and Kir6.2/SUR2B stable line (A) and Kir6.1/SUR2B transient transfection (B) and Kir6.2/SUR2B (stable) (C) are shown with ⁸⁶Rb<sup>+</sup> efflux as % initial content over time (Min).
after a couple of minutes of metabolic poisoning and reached a stable level after 5–8 min. The glibenclamide sensitive currents reversed close to the K$^+$ equilibrium potential of $-90$ mV.

What is the mechanism of metabolic sensitivity? The three major possibilities are illustrated schematically as a cartoon in Figure 3. Initially, a series of experiments were conducted to determine whether PKA and/or adenosine receptors (see Figure 2). Perforated patch clamp whole-cell recording provides further evidence that Kir6.1/SUR2B (A) and Kir6.2/SUR2B (B) are metabolically sensitive. Currents were evoked in asymmetrical K$^+$ conditions (see methods) with 1 s voltage ramp between $-150$ and $+50$ mV from a holding potential of $-80$ mV. [A(i)] and B(ii)] Representative recordings for each channel. [A(ii)] Representative I/V traces for Kir6.1/SUR2B. [A(iii) and B(ii)] Mean current density at a holding potential of $+50$ mV for metabolic inhibition of the Kir6.1/SUR2B and Kir6.2/SUR2B channel currents measured. ***$P < 0.001$ compared to control, (ns) $P > 0.05$ compared to control ($n = 7$ in both).
Supplementary material online, Figure S1) play a role in the response. Mean $^{86}$Rb$^+$ efflux was measured after preincubation with the PKA inhibitors (H-89 and KT5720) and adenosine receptor antagonists (SCH58261 an A2A antagonist and quinoxaline an A1 and A3 antagonist) before the addition of metabolic inhibitors or forskolin. PKA inhibitors reduced efflux observed on the application of forskolin, consistent with our previous observations, but not that induced via the metabolic inhibitors (see Supplementary material online, Figure S1). The adenosine receptor antagonists did not reverse the increased efflux induced by metabolic inhibitors or forskolin (see Supplementary material online, Figure S1). Thus, in this experimental setting, activation of PKA was not responsible for Kir6.1/SUR2B channel activation during metabolic inhibition.

### 3.2 Kir6.1 is not the metabolic sensor

We decided to focus on the role that each of the channel subunits may play. It has been shown that Kir6.2ΔC26 produces currents in the absence of the SUR subunit sensitive to inhibition by ATP. Truncation of the C-terminus (Kir6.1ΔC48/ΔC61) removes the corresponding RKR motif in Kir6.1 and based on previous studies with Kir6.2 it was presumed that this would allow independent membrane expression of the pore. In addition, we combined this with truncation of the N-terminus (ΔN13) as previous observations have shown that progressive deletion of the N-terminus increased basal single-channel open probability. The inclusion of N-terminal deletion might increase our chances of detecting Kir6.1 activity in the absence of SUR. Thus, various ΔN-Kir6.1ΔC mutants were generated and the surface expression and function investigated. Immunofluorescent staining was performed in permeabilized and non-permeabilized cells (Figure 4A). All mutants containing truncation of the C-terminus were independently surface expressed. Kir6.1 and Kir6.1ΔN13 were surface expressed only in the presence of SUR2B (cell permeabilization revealed the presence of channel protein in the absence of the SUR, not shown). Since independent surface expression of the pore was achieved, channel functionality was investigated using $^{86}$Rb$^+$ efflux where the truncated mutants were expressed both with (Figure 4B) and without (Figure 4C) SUR2B. Deletion of the C-terminal 48 or 61 amino acids and/or the N-terminal 13 amino acids did not compromise channel response to pharmacological agents or metabolic poisoning. Two of these mutants, Kir6.1ΔC48 and Kir6.1ΔN13/ΔC48, were further investigated in the absence of co-expression of the SUR. Neither of these demonstrated a greater efflux in the presence of metabolic inhibitors.

We noted enhanced basal efflux after lane expression of Kir6.1ΔC48 and Kir6.1ΔN13/ΔC48 and this was probed further (Figure 5). Using inhibitors that bind to the pore of the channel, PNU-37883A and Ba$^{2+}$, we could inhibit the basal efflux present when truncated Kir6.1 was expressed in the absence of SUR. However, despite the obvious basal channel activity, metabolic inhibition did not increase this efflux further in any of the Kir6.1 mutants (Figures 4 and 5). The lack of metabolic sensitivity of Kir6.1ΔN13/ΔC48 observed in Figure 4C was investigated using the perforated-patch clamp technique. We found basal whole-cell currents inhibited by pore blockade and no increase of these currents with metabolic poisoning (Figure 5B). Finally, we showed that the pore subunit of Kir6.2 did indeed have intrinsic metabolic sensitivity in our assay system. The expression of Kir6.2ΔC26 generated a significant $^{86}$Rb$^+$ flux with metabolic poisoning that was inhibited by Ba$^{2+}$ (Figure 5C). We can conclude that Kir6.1ΔC48, Kir6.1ΔC61, Kir6.1ΔN13/ΔC61, and Kir6.1ΔN13/ΔC48 display basal activity in the absence of SUR2B coexpression, but they are not intrinsically metabolically sensitive in contrast to Kir6.2ΔC26.

### 3.3 The nucleotide-binding domains of SUR2B underlie the metabolic sensitivity

One hypothesis is that the metabolic sensitivity of the Kir6.1/SUR2B complex is accounted for by the binding and hydrolysis of adenine nucleotides at the NBD in SUR2B. Thus, we mutated one or both lysines in the Walker A (Wα) motifs in SUR2B: SUR2BK708A, SUR2BK1349M, and SUR2BK708A/K1349M. The single-point mutations Kir6.1/SUR2BK708A and Kir6.1/SUR2BK1349M both responded with significantly increased $^{86}$Rb$^+$ efflux to metabolic poisoning (Figure 6). In contrast, SUR2BK708A/K1349M when expressed with Kir6.1 was essentially unresponsive to metabolic poisoning and levcromakalim. In contrast, Kir6.2/SUR2BK708A/K1349M showed increased $^{86}$Rb$^+$ efflux on the addition of levcromakalim and metabolic inhibitors. We further investigated the role of NBD2 as this has been thought to be of crucial importance in the regulation of channel activity by intracellular MgADP. Residues G1443 and G1449 are situated within the 'linker' region of NBD2 which is proposed to couple nucleotide hydrolysis to down-stream actions in several ABC proteins, and when these were mutated to aspartic acid in SUR1, they failed to respond to activation by diazoxide and stimulation by MgADP. Both mutations were introduced into SUR2B and expressed individually with Kir6.1 and Kir6.2. The reconstituted $K_{ATP}$ channels showed normal responses indistinguishable from control in $^{86}$Rb$^+$ efflux assays (see Supplementary material online, Figure S2). Finally, we confirmed that the Wα lysines are of predominant importance in the metabolic regulation of Kir6.1/SUR2B using electrophysiology (Figure 7). Figure 7A confirms that Kir6.2/SUR2BK708A/K1349M is indeed still functional, showing increased currents on the addition of pinacidil and metabolic inhibitors. In contrast, Figure 7B shows that Kir6.1/SUR2B K708A/K1349M is rendered unresponsive to metabolic inhibition.
Figure 4 Truncation mutants of Kir6.1 are surface expressed without SUR2B expression, functional in the presence of SUR2B but do not respond to metabolism in its absence. (A) Surface staining of Kir6.1-HA N- or C-terminal deletion mutants in non-permeabilized CHO-K1 cells, expressed with (left panel) and without (right panel) SUR2B. All images were captured at the same exposure (1 s), using the same objective and were not subject to any other form of image enhancement other than the use of a green look-up table. (B) The effect of truncation of both the N and C terminus of Kir6.1 on ^86Rb$^+$ efflux when coexpressed together with SUR2B. (C) The behaviour of Kir6.1, Kir6.1ΔN13/ΔC61, and Kir6.1ΔC48 expressed in HEK293 cells in the absence of SUR2B. Also shown is ^86Rb$^+$ efflux in untransfected HEK293 cells. The data represent mean ± SEM (n = 9). ***P < 0.001, **P < 0.01 compared to control and not significant (ns) P > 0.05 compared to control.
4. Discussion

4.1 Kir6.1/SUR2B is intrinsically metabolically sensitive

In our study, we have shown that the cloned equivalent of the VSM K\textsubscript{ATP} channel (referred to as K\textsubscript{ATP}/K\textsubscript{NDP} below), Kirk6.1/SUR2B, is inherently sensitive to metabolic perturbations and does not necessarily have to rely on inputs via hormonal signalling pathways and protein kinases for activation. Furthermore, and in contrast to Kir6.2 containing complexes, the SUR2B subunit is the sensor via the action of adenine nucleotides at the NBDs. There is redundancy...
in the apparent function of the NBDs as mutation of both is necessary to abolish this response. It has been well established that KATP/KNDP channels are involved in maintaining vascular tone and in vasodilatation in response to exercise and ischaemia, particularly in the coronary arteries.\cite{4,6,29} The key components appear to be a channel constituted from SUR2 and Kir6.1. Mice with global genetic deletion of either protein suffer from coronary artery vasospasm and sudden death.\cite{17,30} Furthermore, animals and also flies lacking this channel are more likely to succumb to infection once infected due to abnormal vascular reactivity in the coronary circulation.\cite{31,32} It is generally thought that adenosine release from metabolically challenged tissues activates the channel via a PKA-dependent mechanism in the VSMs though there is also evidence that ~50% of the adenosine-mediated dilation may be the endothelium dependent in skeletal muscle.\cite{2} However, a more complex scenario has emerged recently. Transgenic expression of SUR2B selectively in VSMs in the Abcc9 \textit{−/−} mouse did not abrogate the vascular phenotype suggesting that KATP channels outside VSMs, for example in the endothelium or in the peripheral nerve endings, might be important in shaping the response.\cite{13} Furthermore, expression of a dominant negative KATP channel subunit in the endothelium led to increased coronary perfusion pressure due to release of endothelin-1.\cite{34} Thus, KATP channels may be sensing metabolic impairment in a number of different tissues and in a number of different ways in the intact vascular bed. Our demonstration of the intrinsic metabolic sensitivity of the K\textsubscript{ATP}/K\textsubscript{NDP} channel allows for alternative mechanisms upstream of the VSM and the potentiation and/or amplification of the response via hormonal inputs at this level.

4.2 The mechanism of metabolic sensing

How in molecular terms do K\textsubscript{ATP}/K\textsubscript{NDP} channels directly sense metabolism? In Kir6.2 containing channels, the focus has been on the pore forming subunit. The truncation of the C-terminus allows the expression of Kir6.2 without SUR and this channel is activated by a reduction in ATP levels.\cite{27} Key residues mediating ATP inhibition and binding have been elucidated and a molecular model has been developed.\cite{11,35} In contrast, our data show that Kir6.1 does not have this ability to sense changes in cellular adenine nucleotides. In Kir6.2, three key residues (R50, K185, and R201) are thought to complex the phosphates in ATP\textsuperscript{11} and in Kir6.1 the analogous residues are identical apart from the conservative substitution (R195 in Kir6.1 for K185 in Kir6.2). The differential ATP sensitivity is likely accounted for by a subtle change in the fold of this domain. In Kir6.1/SUR2B, it is the SUR that underlies the metabolic sensitivity. These observations also raise the question of whether and how SUR might modulate metabolic sensitivity in Kir6.2 containing channels.\cite{36} Furthermore, it would make sense if these channels were sensitive to the metabolic product ([ATP]/[ADP][P]) as [ATP] may be relatively well buffered, for example, during early ischaemia in cardiac cells.\cite{37} Indeed, it remains a possibility that the ATP-binding domain of Kir6.2 may not be the major metabolic sensor and that changes of adenine nucleotides detected at the NBD in SUR might be more physiologically important. Other investigators have suggested that Kir6.1 has similar ATP sensitivity to Kir6.2 under some circumstances; however, these experiments were performed in the presence of SUR and if SUR regulates access to or has an allosteric influence on that ATP-binding site then our results are not necessarily discrepant.\cite{20}

The obvious molecular sensors in SUR2B are the NBDs. In particular, they are known to mediate channel activation with increasing MgADP concentrations in inside-out patches.\cite{14,28} The NBDs in ABC transporters actually function as a dimer\cite{38} and ATP binding at NBD1 and MgADP binding at NBD2 are essential for nucleotide activation of SUR1.\cite{39} Our data show that it is necessary to mutate W\textsubscript{A} lysines in both NBDs in SUR2B to completely abrogate the functional response to metabolic poisoning within the Kir6.1/SUR2B complex since individual mutation of the W\textsubscript{A} lysine of either NBD1 or NBD2 of SUR2B did not affect gross channel function. In addition, linker mutations in NBD2 in SUR2B are also functional in intact cells. Furthermore, both metabolic poisoning and potassium channel openers activate Kir6.2 in complex with SUR2BK708A/K1349M. These data are incongruent to some extent with previous literature;\cite{14,28} however, the behaviour in intact cells with SUR2B might be different from that in inside-out patches with SUR1. For example, there is evidence for differential nucleotide binding affinities between the different SUR isoforms.\cite{40} Kir6.1 rendered non-functional when expressed in combination with SUR2BK708A/K1349M? This seems unlikely as the double mutant is functional in combination with Kir6.2 and can thus chaperone a Kir6.x subunit to the plasma membrane. In addition, Kir6.1 mutants that allow surface trafficking in the absence of SUR generate basal currents. It is also implies that some of the basal activity of Kir6.1 is suppressed by interaction with SUR.\cite{20}

Potassium channel openers bind to transmembrane domains 13–16 and, in particular, to a cytosolic loop
between 13–14 and determinants in helices 15–16.\textsuperscript{41,42}
However, despite the different binding sites, there is a strong functional dependency between $K_{\text{ATP}}$ channel opener action and nucleotide diphosphate activation.\textsuperscript{43,44}

4.3 Features that modulate the response
In HEK293 cells, it was necessary to inhibit both oxidative metabolism and glycolysis before channel activation occurred and in VSMs similar observations have been made.\textsuperscript{45} This suggests that one pathway could compensate for the other in ATP provision over a relatively short-time frame. There is evidence in cardiac cells that the $K_{\text{ATP}}$ channel is preferentially regulated by glycolytic ATP and that phosphorelay networks and creatine kinase are important in the shuttling of ATP from mitochondria to the plasma membrane.\textsuperscript{46,47}

Figure 7  Mutation of both $W_6$ motifs abolishes the response of Kir6.1/SUR2B but not Kir6.2/SUR2B to metabolic inhibition in whole-cell perforated patch recordings. (A) Kir6.2/SUR2B/K708A/K1349M is functional. Currents were evoked using a 1 s voltage ramp between $-150$ and $+50$ mV from a holding potential of $-80$ mV. (i) Representative traces for each of the conditions. (ii) Representative current–voltage relationships. (iii) Current/density bar chart showing the mean data ($n = 6$). (B) Kir6.1/SUR2B/K708A/K1349M does not respond to metabolic inhibition. (i) Representative traces for each of the conditions. (ii) Current/density bar chart showing the mean data. ***$P < 0.001$ compared to control, (ns) $P > 0.05$ compared to control.
4.4 Conclusion
We have shown that the cloned equivalent of the vascular 
K\textsubscript{ATP}/K\textsubscript{GDP} channel has intrinsic sensitivity to metabolism. 
This property is endowed by the likely processing of 
adrenaline nucleotides at the NBD of SUR2B. In our studies, 
both NBD1 and NBD2 seem equivalent and inactivation of 
both is necessary to completely abrogate the response.

Supplementary material
Supplementary Material is available at Cardiovascular 
Research online.

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