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Phenformin has a direct inhibitory effect on the ATP-sensitive potassium channel

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ABSTRACT

The biguanides, phenformin and metformin, are used in the treatment of type II diabetes mellitus, as well as being routinely used in studies investigating AMPK activity. We used the patch-clamp technique and rubidium flux assays to determine the role of these drugs in ATP-sensitive K^+ channel (K_{ATP}) regulation in cell lines expressing the cloned components of K_{ATP} and the current natively expressed in vascular smooth muscle cells (VSMCs). Phenformin but not metformin inhibits a number of variants of K_{ATP} including the cloned equivalents of currents present in vascular and non-vascular smooth muscle (Kir6.1/SUR2B and Kir6.2/SUR2B) and pancreatic β-cells (Kir6.2/SUR1). However it does not inhibit the current potentially present in cardiac myocytes (Kir6.2/SUR2A). The highest affinity interaction is seen with Kir6.1/SUR2B (IC_{50} = 0.55 mM) and it also inhibits the current in native vascular smooth muscle cells. The extent and rate of inhibition are similar to that seen with the known K_{ATP} blocker PNU 37883A. Additionally, phenformin inhibited the current elicited through the Kir6.2ΔC26 (functional without SUR) channel with an IC_{50} of 1.78 mM. Phenformin reduced the open probability of Kir6.1/SUR2B channels by ~90% in inside-out patches. These findings suggest that phenformin interacts directly with the pore-forming Kir6.0 subunit however the sulphonylurea receptor is able to significantly modulate the affinity. It is likely to block from the intracellular side of the channel in a manner analogous to that of PNU 37883A.

1. Introduction

K_{ATP} channels are present in many cell types and couple cell metabolism to potassium flux across cell membranes (Rodrigo and Standen, 2005). Metabolic regulation of K_{ATP} is mediated via cellular changes in ATP, where increased or decreased ATP concentrations result in inhibition and activation of K_{ATP}, respectively. In addition, rising ADP as a result of a metabolic challenge such as ischaemia and hypoxia also activate the channel (Rodrigo and Standen, 2005).

K_{ATP} channels are composed of an octameric complex of pore-forming subunits (Kir6.1 or Kir6.2), members of the inwardly rectifying potassium channel family, and the sulphonylurea receptor subunit (SUR1, SUR2A and SUR2B) who are members of the ATP-binding cassette family of proteins (Seino, 1999; Rodrigo and Standen, 2005). Co-assembly of a particular Kir6 with a particular SUR in different tissues generates currents that have a characteristic conductance, nucleotide regulation and pharmacology (Seino, 1999; Babenko et al., 1998; Tucker and Ashcroft, 1998). For example, Kir6.1 with SUR2B is thought to comprise the vascular smooth muscle K_{ATP} current (Yamada et al., 1997; Beech et al., 1993; Cui et al., 2002; Miki et al., 2002), Kir6.2 with SUR2B is present in non-vascular smooth muscle (Isomoto et al., 1996), Kir6.2 with SUR2A or SUR1 comprise the cardiac K_{ATP} channel (Alekseev et al., 2005) and the pancreatic K_{ATP} channel respectively (Aguilar-Bryan et al., 1995; Inagaki et al., 1995).

Oral biguanides and metformin in particular are used in the treatment of type II diabetes mellitus. Phenformin was withdrawn from clinical use in the 1970s as it was associated with fatal lactic acidosis. They act by sensitising peripheral tissues to insulin and inhibiting hepatic gluconeogenesis. Furthermore, metformin and phenformin are used as activators of AMP-activated protein kinase (AMPK) and a number of their cellular effects are probably mediated through this action (Zhou et al., 2001). Indeed, these drugs are routinely used to investigate AMPK involvement in cell signalling (Hardie, 2008). AMPK, a serine/threonine kinase, coordinates cellular energy metabolism and ATP synthesis/conservation with metabolic demand to regulate the energy balance within the cell (Hardie and Carling, 1997). It is activated by an increased AMP:ATP ratio and subsequently acts on various downstream targets (Hardie, 2004). It has been reported that AMPK can couple membrane transport with cellular metabolism by inhibiting some ion transporters such as cystic fibrosis transmembrane regulator (CFTR) and the epithelial sodium channel in order to promote cell survival (Hallows et al., 2003; Woollhead et al., 2005). More recently, the presence of AMPK has been shown to promote surface expression of cardiac K_{ATP} channels and to be important during preconditioning of cardiomyocytes (Sukhodub et al., 2007).

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As both AMPK and K_{ATP} channels are activated by changing ATP and AMP: ATP ratios, is it possible that K_{ATP} is a downstream target of AMPK? In this study we investigate the actions of metformin and phenformin on K_{ATP} channels. We show that phenformin has a direct effect on the K_{ATP} channel independent of AMPK at concentrations commonly used to study AMPK.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Aldrich (Poole, UK) except levcromakalin (Tocris, UK).

2.2. Cell culture

Human Embryonic Kidney (HEK) 293 cells stably transfected with Kir6.1/SUR2B, Kir6.2/SUR2B, Kir6.2/SUR2A and Kir6.2/SUR1 were maintained in G418 and Zeocin selective media as previously described (Cui et al., 2001; Giblin et al., 1999). Transfection of Kir6.2ΔC26 was carried out using FuGENE HD (Roche Diagnostics, UK) as per the manufacturers' instructions. GFP (100 ng) was co-transfected to enable transfection success and efficiency to be assessed and cells were patched 48 h after transfection.

2.3. Rubidium flux

A $^{86}Rb^{+}$ assay was used to determine amount of flux passing through the K_{ATP} channels in response to various drugs as previously described (Muzyamba et al., 2007; Farzaneh and Tinker, 2008). The cells (in 6-well dishes) were incubated for 24 h with $^{86}RbCl$ (0.037 MBq/mL) before being washed three times with HBS assay medium (10 mM HEPES, pH 7.4, 10 mM glucose, 130 mM NaCl, 7 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$). Cells were then pre-incubated with 2 mL of HBS ± 10 mM phenformin or metformin (concentration commonly used to activate AMPK) for 30 min at 25 °C before channel activators/inhibitors were added as follows (DMSO, 10 μM levcromakalin, 10 μM levcromakalim + 10 μM glibenclamide). After a further 15 minute incubation at 25 °C, the supernatant was aspirated into vials and the cells were lysed using HBS + 2% triton solution and collected. All vials were assayed for ^{86}Rb content by measurement of Cherenkov radiation in a liquid scintillation counter (TriCarb, Packard 2000CA). Efflux was expressed as a percentage relative to the total amount of radioactivity incorporated. One-way ANOVA followed by a Bonferroni test was use to determine statistical significance as indicated in the text.

2.4. Isolation of aortic smooth muscle cells

Mouse aortic smooth muscle cells were isolated from C57BL/6 male or female mice that were 6 to 8 weeks old. The animals were allowed free access to standard chow and water *ad libitum*. The animals were housed in a 12 hour light–dark cycle in a temperature controlled environment (21 °C). The work conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the UCL Animal Ethics Committee and performed in accordance with British Home Office regulations and guidance (project licence PPL\6732). Mice were killed by isoflurane anaesthesia. The aorta was isolated and cleaned of fat and connective tissue and placed in phosphate buffered saline (PBS) solution. The lumen was cut through and the endothelium removed. Endothelium denuded whole tissue was placed in a sterile low calcium dissociation medium (containing 110 mM NaCl, 5 mM KCl, 0.16 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.5 mM NaH_2PO_4, 10 mM Glucose, 10 mM HEPES, 0.49 mM EDTA, 10 mM Taurine, 0.037 mM Phenol Red, pH 7.4 with NaOH) bubbled with 95% O_2/5% CO_2 additionally containing 0.95 mg/mL Papain, 0.2% fatty acid free BSA, 0.5 mM DTT. The tissue was incubated with shaking at 37 °C for 50 min before being washed in dissociation medium alone. Isolated cells were finally triturated using a wide-bore fire polished Pasteur pipette and stored at 4 °C until use.

2.5. Patch clamping

Whole-cell patch-clamp recordings were performed as previously described (Quinn et al., 2003). Capacitance transients and series resistance in whole-cell recordings was compensated electronically by using amplifier circuitry (Axopatch 200B). Data were filtered at 1 kHz using the filter provided with the Axopatch 200B (4 pole Bessel) and sampled at 5 kHz using a Digidata 1440 (Axon Instruments). Currents were acquired and analysed using pClamp10 (Axon Instruments). Pipette solutions (whole-cell and inside-out) contained (mM); 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA and 5 HEPES, pH 7.2 using KOH. For whole-cell recordings the pipette solution was supplemented with 1 mM MgATP and 0.5 mM NaUDP. In whole-cell studies the bath solution contained (mM); 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$ and 5 HEPES (pH 7.4). Whole-cell recordings from mouse aortic smooth muscle cells were carried out in bath solution containing (mM); 110 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 15 $NaHCO_3$, 0.5 KH_2PO4, 0.5 NaH_2PO4, 10 Glucose, 10 HEPES (pH 7.2). For perforated patch experiments the bath solution contained (mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5 HEPES (pH 7.4). A stock solution of amphotericin B in dimethyl sulphoxide (DMSO) was prepared and diluted in a standard pipette solution to give a final concentration of 200 μg/mL. For inside-out recordings the bath solution contained (mM) 140 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES (pH 7.2). Pipette resistances were between 2 and 4 mΩ for whole-cell recordings and 5–8 mΩ for single-channel recordings. Agents were applied to the bath using a gravity-driven system. Single-channel open probability (NPo) was determined using Clampfit 10.0.

2.6. Data analysis

Concentration–response curves were analysed and fitted using the sigmoidal fitting function in Origin version 6. This function allows the calculation of the drug concentration at which half-maximal inhibition (IC_{50}) takes place according to the following Eq. (1) when the *X*-axis is set to a logarithmic scale:

$$\frac{I}{I_{Glib}} = \frac{A_1 - A_2}{1 + (X/IC_{50})^k} + A_2 \quad (1)$$

Where I/I_{Glib} is the inhibitory effect measured relative to the glibenclamide-sensitive current at −50 mV, *X* is the drug concentration, A_1 is the initial *Y* value, A_2 is the final *Y* value and *k* is the slope factor.

To analyse the rate of current inhibition, time-course data was also fitted using the equation (in Origin 6).

$$Inhibition = \frac{A_1 - A_2}{1 + e^{(x - x_0)/k}} + A_2 \quad (2)$$

Where *x* is the time at any given point, x_0 is the time at no inhibition and *k* is the time constant. Other parameters are as described for Eq. (1).

Statistical analysis was carried out using one-way ANOVA with a Bonferroni post-hoc test or a paired Student's *t*-test as appropriate. Statistical significance is indicated in the figure legends. Data are presented as mean ± S.E.M. Where appropriate, current density is presented normalised to the control.

3. Results

3.1. Effects of phenformin and metformin on K_{ATP} current

Initially, we were interested in exploring the role of AMPK in the regulation of K_{ATP} channels. Phenformin and metformin have been shown to result in the activation of AMPK and we used them in an ^{86}Rb flux assay where we could monitor the passage of Rb through the channels as a potassium surrogate in response to various activators or inhibitors. Initially, we used HEK 293 cells that were stably transfected with different Kir6.0 and SUR subunits. During metabolic poisoning we noticed that pre-application of phenformin considerably reduced the flux compared to control (not shown). In order to remove the complication of potential changes in cellular nucleotides with metabolic poisoning we activated the channel directly with a specific potassium channel opener (KCO), levcromakalim. Under these conditions, phenformin (18.19 ± 0.57%, $n = 9$, $P < 0.001$) but not metformin (27.11 ± 1.47%, $n = 9$, $P > 0.05$) inhibited flux activated by 10 μM levcromakalim (28.36 ± 0.43%) to control levels (17.81 ± 0.46%) in the Kir6.1/SUR2B cell line (Fig. 1A). However, phenformin (83.4 ± 2.77% compared to 81.42 ± 0.75 with levcromakalim, $n = 9$, $P > 0.05$) did not inhibit the flux via Kir6.2/SUR2B in this assay (Fig. 1B, but see below). In both cell lines, 10 μM glibenclamide inhibited levcromakalim-activated flux to basal levels ($n = 9$, $P < 0.001$). Next it was investigated whether or not analogous effects were also observed in intact cells using the perforated patch-clamp technique. Current was elicited by a repetitive 1 s voltage ramp from −150 mV to +50 mV every 15 s. Fig. 2 shows that the application of 10 mM metformin did not induce a significant inhibition of the levcromakalim-activated current (at +50 mV, control: 69.52 ± 7.9; levcromakalim: 173.49 ± 24.76; metformin: 166.74 ± 30.59 pA/pF, $n = 8$, $P < 0.05$ when compared to control). However, the subsequent perfusion of 10 mM phenformin reduced current to control levels (75.28 ± 18.43 pA/pF, $n = 8$, $P > 0.05$ compared to control) (Fig. 2A and B). 10 μM glibenclamide reduced levcromakalim-activated current to 55.48 ± 8.53 pA/pF ($n = 8$, $P > 0.05$ compared to control). This suggests that the biguanides, metformin and phenformin are acting differentially on the Kir6.1/SUR2B channel. The inhibition induced by phenformin did not show prominent voltage-dependence as shown by the voltage ramp traces in Fig. 2C.

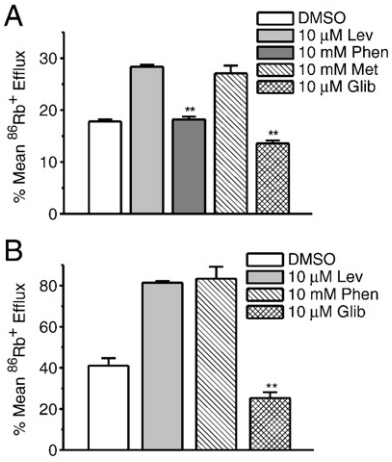

Fig. 1. Effect of phenformin and metformin on different types of K_{ATP} channels. (A) HEK293 cells stably transfected with Kir6.1/SUR2B were pre-incubated with HBS ± phenformin (phen) or metformin (met) before addition of levcromakalim (lev) or levcromakalim + glibenclamide (glib). Mean $^{86}Rb^+$ flux was calculated as % efflux of initial $^{86}Rb^+$ content. Data are shown as mean ± S.E.M, $n = 9$. $^{**}P < 0.001$ and when compared to the lev. (B) HEK293 cells stably transfected with Kir6.2/SUR2B were pre-incubated with HBS ± phenformin (phen) before addition of levcromakalim (lev) or levcromakalim + glibenclamide (glib). Mean $^{86}Rb^+$ flux was calculated as % efflux of initial $^{86}Rb^+$ content. Data are shown as mean ± S.E.M, $n = 9$. $^{**}P < 0.001$ and when compared to the lev.

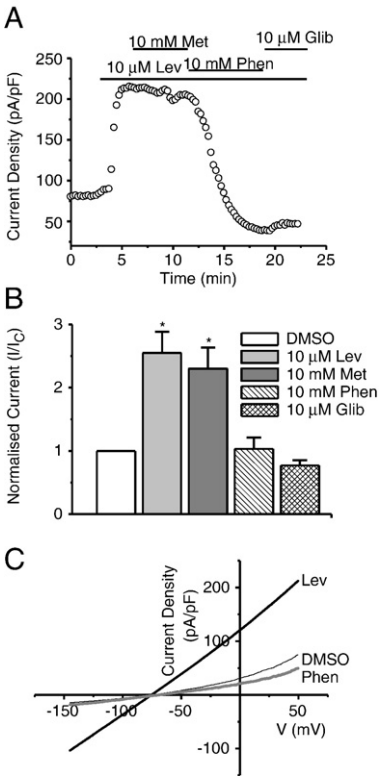

Fig. 2. The AMPK activator phenformin but not metformin inhibits whole-cell K_{ATP} currents elicited through Kir6.1/SUR2B. (A) Time-course (at +50 mV) of phenformin (phen) inhibition of currents recorded from a perforated patch. Currents were evoked using a series of ramps (1 s every 15 s) from −150 mV to +50 mV from holding potential of −80 mV. (B) Mean perforated patch data taken at +50 mV and normalised to the control (DMSO) ($n = 8$). $^{*}P < 0.05$ compared with control. (C) Voltage ramps recorded between −150 mV and 50 mV (1 s in length) under the conditions indicated.

3.2. Effects of phenformin on other K_{ATP} channel subtypes

The standard whole-cell patch-clamp configuration was used to investigate whether the observed effect of phenformin on the Kir6.1/SUR2B channel was the same with different Kir6.0 and different SUR subunits (Fig. 3). Whole-cell recordings for Kir6.2/SUR2B (Fig. 3A and B) and Kir6.2/SUR1 (Fig. 3E and F) show that in the presence of a potassium channel opener such as pinacidil or diazoxide, application of phenformin results in current inhibition. However, data in Fig. 3C and D show that phenformin has no effect on Kir6.2/SUR2A currents (at −60 mV, control: −29.15 ± 3.74; 10 μM pinacidil: −192.05 ± 26.66; 10 mM phenformin: −210.77 ± 23.03; 10 μM glibenclamide: −54.44 ± 12.16, $n = 10$, $P < 0.005$ compared to control). Comparison of the extent of inhibition by phenformin of the KCO-evoked current through the different Kir6/SUR combinations (Fig. 4A) showed that the different channels were inhibited to varying degrees. Kir6.1/SUR2B was inhibited by 97 ± 2.0% whereas Kir6.2/SUR2A shows little inhibition in the presence of phenformin (1.8 ± 9.3%). Kir6.2/SUR2B and Kir6.2/SUR1 are inhibited by 59.9 ± 8.5% and 67.6 ± 9.5%, respectively. The flux data did not show an inhibitory effect of phenformin on Kir6.2/SUR2B however our experience with this assay is that it is not as sensitive as the patch-clamp technique and is more variable in reporting intermediate effects.

Dose–response curves for phenformin using the different Kir6/SUR combinations show that the IC_{50} for Kir6.1/SUR2B is 550 μM whereas the IC_{50} for Kir6.2/SUR1 is 9.33 mM. Interestingly, when these experiments were repeated using a Kir6.2 C-terminal truncation mutant (Kir6.2ΔC26) which allows for the Kir6.2 subunit to be functionally expressed at the membrane in the absence of SURs (Tucker et al., 1997), phenformin inhibition was clearly observed. The

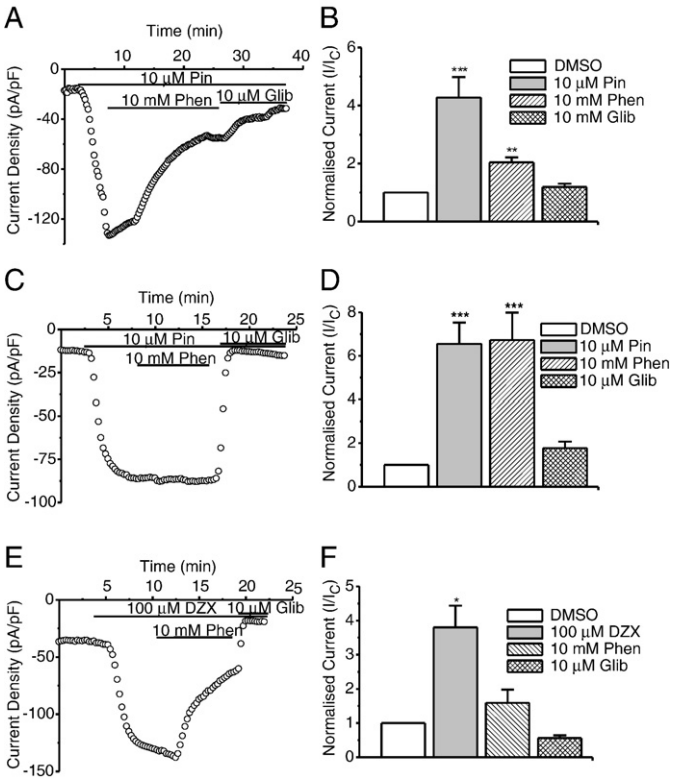

Fig. 3. Effect of phenformin on different K_{ATP} currents in HEK cells. (A) Whole-cell recording from HEK 293 cell stably transfected with Kir6.2/SUR2B. Currents were evoked using a series of 1 s voltage ramps from −100 mV to +100 mV from a holding potential of 0 mV every 15 s. K_{ATP} current was activated by superfusing the patch with 10 μM pinacidil, 10 mM phenformin and 10 μM glibenclamide were superfused in the presence of pinacidil as indicated by the solid bars. (B) Normalised mean whole-cell data for Kir6.2/SUR2B taken at −60 mV ($n=8$) in the presence and absence (DMSO) of phenformin. (C) Time-course of currents through Kir6.2/SUR2A channels, activated by 10 μM pinacidil and superfused with 10 mM phenformin or 10 μM glibenclamide. (D) Normalised mean Kir6.2/SUR2A currents at −60 mV in the presence and absence (DMSO) of Phenformin ($n=10$). (E) Representative time-course trace for Kir6.2/SUR1. Current was activated at −60 mV by superfusing with 100 μM diazoxide and inhibited with 10 mM phenformin and 10 μM Glibenclamide. (F) Normalised mean whole-cell current from Kir6.2/SUR1 channels ($n=4$) in the presence and absence of phenformin. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ when compared to the control.

IC_{50} of this inhibition was 1.78 mM, in between that of Kir6.1/SUR2B and Kir6.2/SUR1 (Fig. 4B). Furthermore, the rates of inhibition of Kir6.2ΔC26 (0.53 ± 0.11 min) and Kir6.1/SUR2B (0.71 ± 0.15 min) current by phenformin are comparable (Fig. 4D). Conversely, the rates of inhibition of Kir6.2/SUR2B and Kir6.2/SUR1 were slower at 3.12 ± 0.63 min and 1.18 ± 0.21 min, respectively (Fig. 4C and D). This indicates that phenformin can act directly on the Kir6 subunit and that the SUR subunits may modulate its' affinity for the pore-forming subunit.

3.3. Phenformin acts on the pore-forming Kir6 subunit

To further examine whether phenformin acts directly on the pore-forming subunit, the extent and rates of inhibition between phenformin and a known pore blocker of K_{ATP}, PNU 37883A (Cui et al., 2003) were directly compared. Whole-cell patch-clamp recordings from HEK293 cells stably transfected with Kir6.1/SUR2B showed that the percentage inhibition of levcromakalim-activated current observed with PNU 37883A (81.89 ± 3.32%) and phenformin (95.91 ± 2.51%) is similar at the concentration used (Fig. 5A and B). In addition, there is no significant difference in the rate of inhibition between PNU 37883A and phenformin (0.57 ± 0.16 min and 0.71 ± 0.15 min respectively, $n=8$, $P>0.05$) (Fig. 5C). This indicates that phenformin can act as

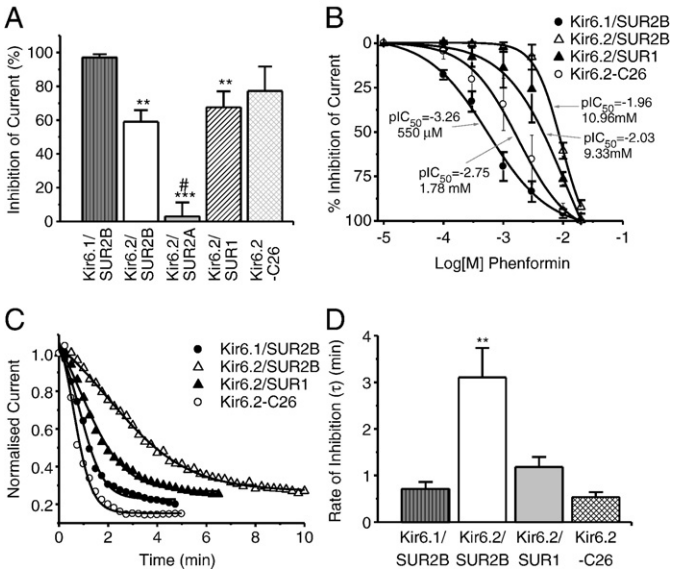

Fig. 4. Extent and rate of inhibition of different K_{ATP} currents by phenformin. (A) Mean phenformin-induced percentage inhibition of the different KATP currents as indicated ($n=4$–10). (B) Mean phenformin dose–response relationships for Kir6.1/SUR2B, Kir6.2/SUR2B, Kir6.1/SUR1 and Kir6.2deltaC26. The lines are the best fits of the data to Eq. (1). Inhibition by phenformin was calculated as the percentage inhibition of 10 μM glibenclamide-sensitive current. Data are mean S.E.M., $n=4$–10. (C) Normalised representative traces of current inhibition by 10 mM phenformin for the indicated channel types. (D) Mean rate of inhibition of the different K_{ATP} currents. Rates were calculated from fits of Eq. (2) to individual time-course traces as those shown in C, $n=4$–10. $^{***}P<0.0001$, $^{**}P<0.001$ compared to Kir6.1/SUR2B. $\#P<0.0001$ when compared to Kir6.2/SUR1 or Kir6.2/SUR2B.

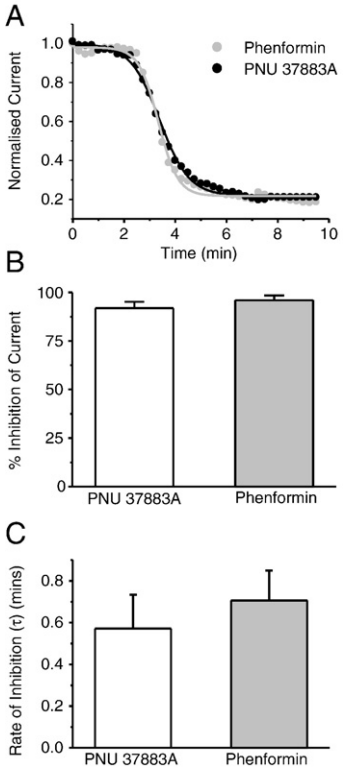

Fig. 5. Comparison of the effect of phenformin with that of the pore blocker PNU 37883A on Kir6.1/SUR2B in HEK293 cells. (A) Representative time-course traces for phenformin and PNU 37883A. Data is normalised to the maximum current induced by 10 μM levcromakalim. (B) Mean % inhibition of current in the presence of 10 mM phenformin and 100 μM PNU 37883A, $n=8$. (C) Mean rate of inhibition of current with phenformin and PNU 37883A, $n=8$.

quickly as the pore blocker PNU 37883A to inhibit the Kir6.1/SUR2B channel. It is thought that PNU 37883A acts from the cytoplasmic side of the membrane and some of the delay is due to the drug crossing the membrane.

3.4. Inhibition of the native VSM K_{ATP} current by phenformin

Is this effect of phenformin unique to the stable lines or is a similar phenomenon observed using native mouse aortic smooth muscle cells that are thought to contain Kir6.1/SUR2B? In Supplementary data we show a brief characterisation of these cells. Fig. 6A shows a representative time-course plot of a whole-cell recording (taken at +20 mV from holding potential of −80 mV) from a typical VSMC from mouse aorta. Representative current traces are shown on the left hand panel. Currents were activated by levcromakalim and subsequently inhibited by 10 mM phenformin past basal levels. The mean current–voltage relationship in Fig. 6B showed that the channel is inhibited in the presence of both phenformin and glibenclamide and that this block is not voltage-dependent. At +20 mV the mean current density is significantly reduced in the presence of phenformin (with levcromakalim), 3.45 ± 0.38 pA/pF, compared to that observed in the presence of levcromakalim alone, 18.3 ± 3.18 pA/pF ($n = 6$, $P < 0.001$) (Fig. 6C). Comparison of the effects of PNU 37883A and phenformin showed that both inhibited the native channel to a similar extent (Fig. 6D and E). The percentage inhibition observed with PNU 37883A was 71.1 ± 2.5% compared to 69.7 ± 5.6% with phenformin ($n = 6$, $P > 0.05$). Furthermore, the rates of inhibition were also comparable at 0.32 ± 0.15 min for PNU 37883A and 0.39 ± 0.02 min for phenformin ($n = 6$, $P > 0.05$). Thus, it is likely that phenformin is having a direct effect on the K_{ATP} channel in both the stably transfected cell lines and in the native aortic smooth muscle cell where it is thought to consist of Kir6.1/SUR2B.

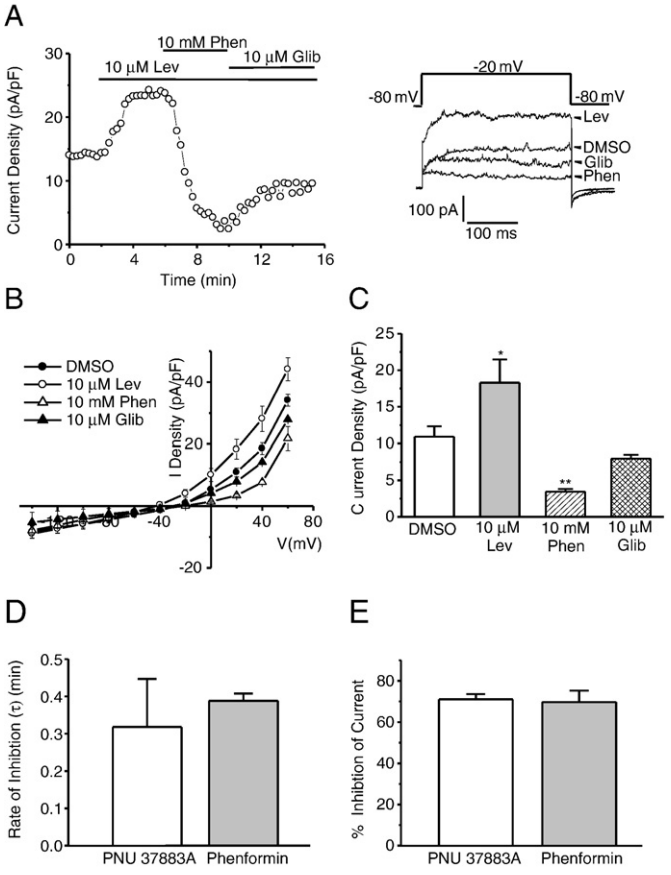

Fig. 6. Effect of phenformin on K_{ATP} current from native mouse aortic smooth muscle cells. (A) Representative time-course trace (left) recorded from a mouse aortic smooth muscle cell. Currents were evoked using a series of repetitive voltage steps from −80 mV to +20 mV as shown on the right-hand trace. Levcromakalim (lev), phenformin (phen) and glibenclamide (glib) were superfused were indicated by the solid bars. The right-hand current trace shows amplitude of currents at +20 mV in the presence of the 3 drugs as indicated by the solid arrows. (B) Current–voltage (I–V) relationships in the presence of levcromakalim, phenformin and glibenclamide. Currents were elicited by a series of voltage steps from −100 to +60 mV in 20 mV increments ($n = 4$–6). (C) Summary of the mean current density (pA/pF) recorded at +20 mV in the presence of the indicated drugs ($n = 4$–6), $^{*}P < 0.01$ and $^{**}P < 0.001$ when compared to the control (DMSO). (D) and (E) Comparison of the rate of inhibition and % inhibition of K_{ATP} current in the presence of 100 µM PNU 37883A ($n = 4$) and 10 mM phenformin ($n = 6$) respectively.

3.5. Phenformin inhibition of Kir6.1/SUR2B in inside-out patches

It is thought that PNU 37883A acts as a pore blocker at the cytoplasmic face of the channel and this accounts for the kinetics of inhibition (Kovalev et al., 2004). We directly tested whether this might be the case for phenformin by pulling inside-out patches in HEK293 cells expressing Kir6.1\SUR2B. The application of phenformin to the cytoplasmic face of the channel led to prominent inhibition of single-channel current (Fig. 7A). The single-channel current amplitude was not affected at −60 mV (1.95 ± 0.09 pA in UDP versus 1.88 ± 0.08 pA in UDP + phenformin, $n = 4$, $P > 0.05$) and inhibition occurred through an apparent reduction in NPo (0.5 ± 0.04 in UDP compared with 0.05 ± 0.04 in UDP + phenformin, $n = 4$, $P < 0.0001$) (Fig. 7B).

4. Discussion

4.1. Inhibition of K_{ATP} by phenformin but not metformin

The activation of AMPK by the biguanides, metformin and phenformin has been shown to occur as a result of an increase in the cytosolic AMP concentration (Zhang et al., 2007). Both of these drugs are thought to act by inhibiting complex I of the respiratory chain in mitochondria that subsequently causes the AMP levels within the cell to increase (Owen et al., 2000; El Mir et al., 2000). These drugs are commonly used to implicate AMPK signalling in cell signalling and ion

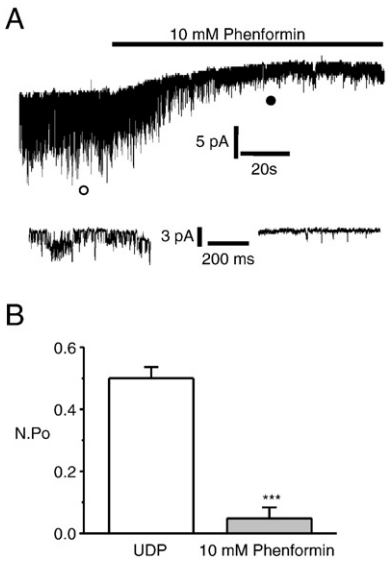

Fig. 7. Effect of phenformin on single-channel current elicited through Kir6.1/SUR2B channels. (A) Representative single-channel trace recorded at −60 mV from an inside-out patch in symmetrical 140 mM K^+ solution. Currents were activated by 3 mM UDP and treated with 10 mM phenformin (indicated by solid bar). Expanded current traces are shown below the main trace from time-points indicated by the empty (UDP) and filled (phenformin) circles, respectively. (B) Bar chart showing mean N.Po values in the presence and absence of 10 mM phenformin ($n = 4$). $^{***}P < 0.0001$ compared with UDP.

transport processes. It has previously been reported for example that the chloride channel CFTR and the sodium channel ENaC, are directly regulated by AMPK (Hallows et al., 2003; Woollhead et al., 2005). Phenformin itself has been identified as an inhibitor of ion transport processes (Saito and Yoshida, 1984). However our data support a direct effect of phenformin on K_{ATP} channels independent of its action on AMPK. Specifically, the discrepancy in the effects of metformin and phenformin at concentrations where they are known to maximally activate AMPK, the relatively rapid effect and the presence of inhibition under whole-cell conditions in which the nucleotide conditions are buffered are highly suggestive. However, most clearly the inhibitory effect on the current is still present in inside-out patches which are devoid of mitochondria.

4.2. Possible mechanism of action by which phenformin inhibits K_{ATP}

Our favoured hypothesis is that phenformin is a pore blocker that partitions into its blocking site either from the membrane or from the cytoplasm. The clear cut block of the truncated Kir6.2 channel, which is missing the last 26 amino acids and can be expressed at the cell membrane as a functional channel, suggests that phenformin is able to interact with the pore (Tucker et al., 1997). The comparable kinetics and magnitude of effect when comparing PNU 37783A, which is thought to act in this fashion, and phenformin is also supportive. However SUR can clearly have a modulatory effect, the presence of SUR2A prevents the inhibition of the channel whereas the presence of SUR2B and SUR1 allow the channel to be inhibited but to different extents. Indeed, we have seen similar phenomena when examining PNU 37883A (Cui et al., 2003). The highest affinity block was for the Kir6.1/SUR2B complex (IC_{50} is 550 μM) the molecular correlate of the vascular smooth muscle K_{ATP} channel (Miki et al., 2002; Cui et al., 2002). We also saw that phenformin could block the native current in aortic vascular smooth muscle cells. We haven't examined the biophysical mechanism in extensive detail but the block appeared to be voltage independent suggesting a blocking site at the periphery of the conduction pathway and it did not reduce the single-channel current amplitude. It appeared to have an apparent effect on channel gating whether through the generation of a blocked single-channel state or through stabilisation of a true closed gating state. It has been demonstrated that PNU 37883A interacts with an 81 amino acid region on the C-terminus of the Kir6.1 subunit (Teramoto, 2006). However the site of potential interaction with phenformin is unknown but it could act in a similar region.

Generally in cell signalling studies metformin and phenformin are used in the 1–10 mM range. At these concentrations Kir6.1\SUR2B would be significantly inhibited. In contrast therapeutic concentrations in patients are in the low μM range (Dell'Aglio et al., 2009; Schulz and Schmoldt, 2003). It may be that the biguanides partition into target tissues to achieve higher local concentrations *in-vivo* if they are acting via AMPK activation. Phenformin is highly lipophilic and has previously been shown to alter the fluidity and the surface charge of phospholipid membranes (Schafer, 1976). Other known AMPK activators and inhibitors have also been shown to be acting through AMPK independent mechanisms. For example, metformin has been proposed to act via an AMPK independent pathway in other cell types including liver and heart (Hawley et al., 2002; Bergheim et al., 2006; Saeedi et al., 2008). In rat hearts it was proposed that metformin action may be mediated by p38 MAPK- and PKC-dependent mechanisms (Saeedi et al., 2008).

A comparison of the structures shows (Fig. 8) that phenformin and metformin differ as the names indicate by the presence of a phenyl group. It is interesting that glibenclamide contains such a group attached to the sulphonyl group. Tolbutamide is even simpler with a simple single phenyl group (not shown) and it is known that these sulphonylureas can interact with the pore as well as the SUR subunit (Gribble et al., 1997). Practically, the pharmacology of related

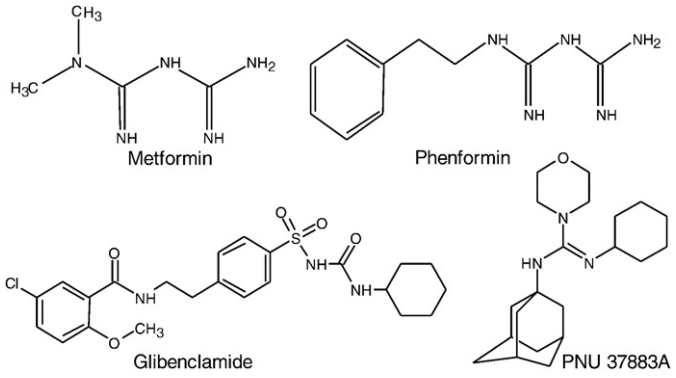

Fig. 8. The chemical structures of phenformin, metformin, glibenclamide and PNU 37883A.

biguanides (and synthetic derivatives) may well be worth reinvestigating in an effort to develop a compound that can both selectively inhibit K_{ATP} channels and activate AMPK promoting both insulin release from pancreatic β-cells and sensitisation to the action of the hormone peripherally. The lack of an effect on the cardiac channel would also be beneficial.

4.3. Conclusions

In conclusion, phenformin has been shown to inhibit K_{ATP} channels to varying extents depending on the complex composition with its action on Kir6.1/SUR2B being the most potent. This is also reflected in native aortic smooth muscle cells that are proposed to contain Kir6.1/SUR2B. It appears to act on the pore but the SUR subunit plays a modulatory role in determining the extent and affinity of inhibition. These effects occur at phenformin concentrations commonly used in cell signalling studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2010.02.023.

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