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Different molecular sites of action for the K_{ATP} channel inhibitors, PNU-99963 and PNU-37883A

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1 We investigated the mechanism of action of two novel nonsulphonylurea ATP-sensitive potassium channel (K_{ATP}) inhibitors, PNU-99963 and PNU-37883A, on four types of cloned K_{ATP} channels.

2 Whole-cell currents were recorded in a symmetrical potassium (140 mM) gradient in HEK-293 cells stably expressing Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B or Kir6.1/SUR2B.

3 PNU-99963 potently inhibited the four K_{ATP} channel clones. The concentration at which half-maximum current was inhibited (IC_{50}) was 66, 41, 43 and 11 nM for Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B, respectively. In contrast, PNU-99963 up to a concentration of 3 μM had no significant effect on current generated in HEK-293 cells by transiently expressing Kir6.2Δ26, a C-terminal truncated pore-forming subunit of Kir6.2.

4 PNU-37883A inhibited four types of K_{ATP} channels, but to different extents. Inhibition of the putative smooth muscle K_{ATP} channel types, Kir6.2/SUR2B (IC_{50}; 15 μM) and Kir6.1/SUR2B (IC_{50}; 6 μM), was significantly greater than inhibition of either the pancreatic β cell or cardiac K_{ATP} channel clones. Moreover, PNU-37883A significantly inhibited currents generated by expressing Kir6.2Δ26 alone, with an IC_{50} of 5 μM, which was significantly increased to 38 μM when Kir6.2Δ26 was expressed with SUR2B.

5 In conclusion, two structurally different nonsulphonylurea compounds, PNU-99963 and PNU-37883A, inhibit K_{ATP} channels via different mechanisms, namely through the sulphonylurea receptor (SUR) and the pore-forming subunits, respectively, although SUR2B reduced the inhibitory effect of PNU-37883A. While PNU-99963 potently inhibits all the four cloned K_{ATP} channels, PNU-37883A has a degree of selectivity towards both smooth muscle K_{ATP} channels, but could not discriminate between them.

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Keywords: ATP-sensitive potassium channel; cloned channels; pore-forming subunit; sulphonylurea receptor; inhibitory sites; PNU-99963; PNU-37883A

Abbreviations: K_{ATP}, ATP-sensitive potassium channel; KCOs, potassium channel opening drugs; NDP, nucleotide diphosphate; SUR, sulphonylurea receptor

Introduction

ATP-sensitive potassium (K_{ATP}) channels are ubiquitously expressed and couple cell metabolism to membrane excitability (Ashcroft, 1988). Classically, these channels are activated by low cytosolic ATP or elevated nucleotide diphosphate (NDP) levels and inhibited by sulphonylurea agents such as glibenclamide and tolbutamide. In addition, K_{ATP} channels can be activated by a structurally diverse group of compounds known as potassium channel opening drugs (KCOs), including agents like levcromakalim and pinacidil. In vascular smooth muscle, KCOs cause membrane hyperpolarisation, increase K^+ efflux and produce smooth muscle relaxation, the effects of which can be inhibited by glibenclamide.

PNU-99963 and PNU-37883A are two novel nonsulphonylurea K_{ATP} channel inhibitors with different structures (Figure 1) which were originally developed on the basis of their weak diuretic effects but subsequently appeared to have some selectivity for the vascular K_{ATP} channel (Humphrey *et al*., 1996). The former is a cyanoguanidine and based on the structure of pinacidil, while the latter is a morpholinoguanidine (Meisheri *et al*., 1993a; Humphrey *et al*., 1996; Khan *et al*., 1997). Early experiments in isolated rabbit mesenteric artery showed that PNU-99963 and PNU-37883A inhibited blood vessel relaxation induced by KCOs, such as pinacidil, levcromakalim and minoxidil sulphate (Meisheri *et al*., 1993a; Khan *et al*., 1997). In addition, PNU-99963 inhibited pinacidil-induced relaxation in different blood vessels from other species, like dog coronary artery and rat aorta (Khan *et al*., 1997). Likewise, *in vivo* experiments showed that PNU-37883A significantly reversed hypotension produced by cromakalim, pinacidil and minoxidil in rats, cats and dogs (Meisheri *et al*., 1993a). Electrophysiological experiments have shown that PNU-37883A at a concentration of 1 – 30 μM selectively inhibited K_{ATP} currents activated by levcromakalim in single smooth muscle cells isolated from rat mesenteric artery, but not K_{ATP} current in cardiac and skeletal myocytes (Wellman *et al*., 1999). However, no data are available with

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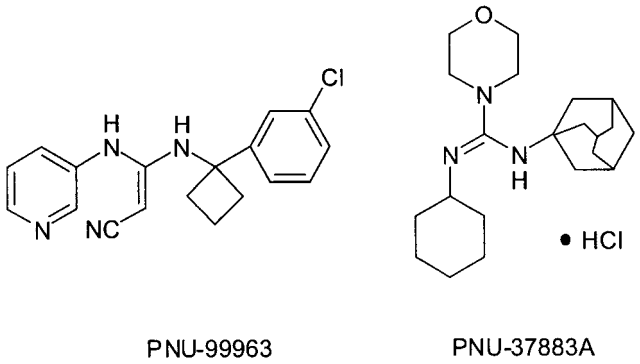

Figure 1 Chemical structure of PNU-99963 and PNU-37883A.

PNU-99963 regarding its direct electrophysiological actions on K_{ATP} currents from any cell type or whether it has any tissue selectivity towards the vasculature.

At the molecular level, the K_{ATP} channel is an octamultimeric complex, which is composed of two subunits: a pore-forming subunit of Kir6.0 subfamily and a sulphonylurea receptor (SUR) (Inagaki *et al*., 1995, 1996; Isomoto *et al*., 1996; Yamada *et al*., 1997; Aguilar-Bryan *et al*., 1998). It has generally been accepted that the pancreatic β cell K_{ATP} channel is composed of Kir6.2 and SUR1, and the cardiac type of Kir6.2 and SUR2A. SUR2B together with either Kir6.2 or Kir6.1 are thought to be the smooth muscle K_{ATP} channels, although these particular cloned K_{ATP} channels cannot fully reconstitute the properties found in some native tissues (Koh *et al*., 1998; Cui *et al*., 2002b). Studies have suggested that different composition of K_{ATP} channels contributes to the different specificity towards the various K_{ATP} channel agents. For instance, tolbutamide inhibits Kir6.2/SUR1 channel but not Kir6.2/SUR2A, while levcromakalim activates Kir6.2/SUR2A but not Kir6.2/SUR1, whereas the opposite is true for diazoxide. These are consistent with results obtained in native pancreatic β cells and cardiac myocytes (Inagaki *et al*., 1995, 1996; Gribble *et al*., 1998). Therefore, cloned channels can be a useful model for studying the tissue selectivity of K_{ATP} channel agents. Recently, PNU-37883A has been shown to inhibit K_{ATP} currents generated by coexpressing either SUR1 or SUR2B with human Kir6.1 in *Xenopus laevis* oocytes, but not the current generated by expressing SUR1 or SUR2B with Kir6.2 (Surah-Narwal *et al*., 1999; Kovalev *et al.,* 2001). These investigators suggested that the major binding site for PNU-37883A might be on Kir6.1 or that Kir6.1 acts allosterically to promote drug binding to SUR1/SUR2B. We therefore designed experiments to more directly address the issue of the site of action of PNU-37883A.

In this study, we first examined the effects of PNU-99963 and PNU-37883A on four types of cloned K_{ATP} channels stably expressed in HEK-293 cells (human embryonic kidney cell line). To further probe the mechanism, we tested the effects of these two compounds on currents generated by Kir6.2Δ26, a C-terminally truncated form of Kir6.2 lacking the last 26 amino acids that is able to express current at the plasma membrane in the absence of SUR (Tucker *et al*., 1997). Our results show that inhibition of K_{ATP} channels by PNU-99963 is primarily through binding to the SUR receptor, while PNU-37883A blocks K_{ATP} channels by acting on the pore-forming subunit of either Kir6.1 or Kir6.2. Preliminary results have been presented to the British Pharmacological Society (Cui *et al.,* 2002a; O'Brien *et al.,* 2002).

Methods

Cell culture and molecular biology

HEK-293 cells were cultured at 37°C under a humidified atmosphere in 5% CO_2 in minimal essential medium with Earle's Salts, L-glutamine, supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (from a stock of 10,000 U ml^{-1} penicillin and 1 mg ml^{-1} streptomycin). Cells were transfected and stable cell lines expressing either Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B or Kir6.1/SUR2B were generated as previously described (Giblin *et al*., 1999; Cui *et al*., 2001).

A truncated form of Kir6.2 (Kir6.2ΔC26), which lacks the C-terminal 26 amino acids, was constructed by introducing a stop codon using mutagenesis (QuickChange Kit, Stratagene, La Jolla, CA, U.S.A.). cDNA of Kir6.2ΔC26 was transiently transfected alone or cotransfected with SUR2B in HEK 293 cells using LipoFECTAMINE (Invitrogen, Paisley, U.K.) according to the manufacturer's instruction. In these experiments, 50 ng of EGFPN1 cDNA (Clontech, Palo Alto, CA, U.S.A.) was also cotransfected to enable transfected cells to be identified by epifluorescence.

Electrophysiology

Currents were recorded in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). Current signals were filtered at 1 – 2 kHz (eight-pole low-pass Bessel) and digitised at 5 kHz using a Digidata 1200 interface and saved onto a computer for later analysis. Patch pipettes from thin-walled (1.5 mm OD) borosilicate glass capillaries (Clark Electromedical, Pangbourne, U.K.) were pulled and fire polished using a DMZ-universal puller (Zietz Instruments, Muenchen, Germany). Pipettes had resistances of 2 – 4 MΩ when filled with electrolyte solution. Electrode capacitance was reduced by coating pipettes with a parafilm/mineral oil suspension and compensated electronically. Series resistance during whole-cell recording was compensated to at least 75% by the amplifier. Cells were bathed in a symmetrical potassium solution and currents were elicited from a holding potential of 0 mV by 100 ms voltage steps in 10 mV increments from – 100 to + 50 mV. Electrophysiological data were analysed using pClamp (version 6; Axon Instruments) and Origin software (Microcal, Northampton, MA, U.S.A.). Current – voltage ($I-V$) relationships were constructed by averaging the current during the last 10 ms of the voltage step. K_{ATP} channel inhibitors were applied in a cumulative fashion and concentration – response curves constructed by calculating the percentage inhibition of the $BaCl_2$-sensitive current measured at – 100 mV in the presence of 10 mM $BaCl_2$.

Data and statistical analysis

Concentration – response curves were analysed and fitted using the Sigmoidal fitting routine in Origin version 6, which when the X-axis is set to a logarithmic scale, calculates the

drug concentration at which half-maximum current is inhibited (IC_{50}) according to the Logistical equation (1):

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

where I/I_{Ba} is the inhibitory effect measured relative to the total $BaCl_2$-sensitive current at -100 mV, X is the drug concentration, A_1 is the initial Y value, A_2 is the final Y value and n is the slope factor.

Values in the text are given as mean ± standard error of the mean (s.e.m.), and n indicates the number of cells. Statistical significance was assessed using one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparison between different groups of cells. P-values <0.05 were considered to be statistically significant.

Solutions and drugs

The standard bath solution contained in mM: 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$ and 5 HEPES (pH 7.4). The pipette solution contained in mM: 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA and 5 HEPES (with 33 mM KOH to pH 7.2). ATP (magnesium salt) and guanidine 5′ diphosphate Na (GDP) were added to the pipette solution at a concentration indicated in the text and the pH of the solutions readjusted. $BaCl_2$, ATP and GDP were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Levcromakalim was a gift from SmithKline Beecham Ltd (Harlow, Essex, U.K.). PNU-99963 was a gift from Pharmacia Corporation (Kalamazoo, Michigan, U.S.A.) and PNU-37883A was either a gift from Pharmacia Corporation or obtained from Biomol (Plymouth Meeting, PA, U.S.A.). PNU-99963 was stored as a 10 mM stock in dimethyl sulphoxide (DMSO) and PNU-37883A stored at 4°C as 20 or 30 mM stocks in a 1 : 4 solution of DMSO and H_2O. On the day of the experiment, stock solutions were diluted in the bath solution to give the desired concentration.

Results

Dialysis of HEK cells with 1 mM ATP in those expressing Kir6.2/SUR1, Kir6.2/SUR2A or Kir6.2/SUR2B caused a time-dependent increase in basal current (Figure 2), consistent with the blockade of K_{ATP} channels by higher concentration of endogenous cytoplasmic ATP before and immediately after establishment of whole-cell recording. In cells expressing Kir6.1/SUR2B, the putative NDP-sensitive potassium channel, current was activated with 1 mM GDP in the pipette solution. In all cases, it took about 5 min for currents to run up to a steady state after the initial establishment of whole-cell recording. The $I-V$ relationships of the expressed currents were essentially linear and could be almost completely blocked by 10 μM glibenclamide (data not shown). Our previous experiments have shown that these stable cell lines produce robust currents with appropriate pharmacological profiles corresponding to the molecular composition of the cloned channels (Giblin *et al*., 1999; 2002; Cui *et al*., 2001).

We first examined the actions of PNU-99963 on currents generated by four types of K_{ATP} channels, Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B. Cells were bathed in a symmetrical potassium (140 mM) solution and currents were elicited by voltage steps from − 100 to + 50 mV at a holding potential of 0 mV. Figure 2 shows that application of 1 μM PNU-99963 markedly inhibited all the four types of cloned currents. Switching to a bath solution containing 10 mM $BaCl_2$, there was a small further decrease in the current at negative potentials, although at positive potentials, currents increased relative to those obtained with PNU-99963, probably reflecting partial wash out of PNU-99963 and the known voltage-dependent block of K_{ATP} currents with $BaCl_2$ (Giblin *et al.,* 1999). The effects of PNU-99963 were reversible, generally taking 5 – 10 min to wash out, although this was not studied in detail. The $I-V$ relations of Kir6.2/SUR and Kir6.1/SUR2B currents measured during the last 10 ms of the voltage steps are shown in Figure 2b. PNU-99963 inhibited currents equally at all potentials and there was no apparent voltage dependence to the block. The mean inhibition current by 100 nM PNU-99963 (measured at − 100 mV and calculated as the percentage block of the total $BaCl_2$-sensitive current) was 58.8 ± 8.60% ($n=4$) for Kir6.2/SUR1, 53.2 ± 7.0% ($n=5$) for Kir6.2/SUR2A, 56.9 ± 7.1% ($n=9$) for Kir6.2/SUR2B and 91.0 ± 2.5% ($n=5$) for Kir6.1/SUR2B. Among the cloned channels, Kir6.1/SUR2B was significantly more sensitive to

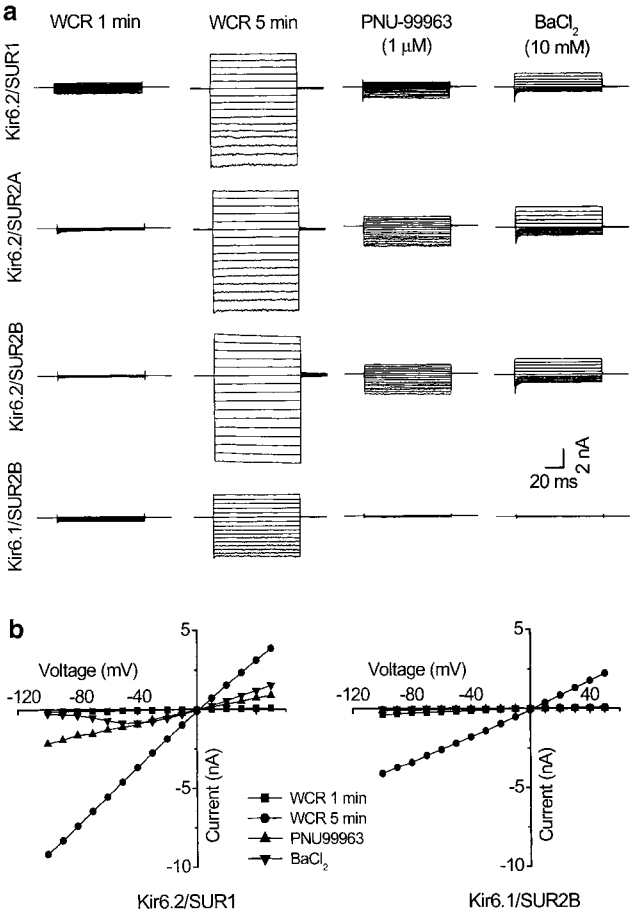

Figure 2 Effects of PNU-99963 on Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B currents stably expressed in HEK293 cells. (a) Whole-cell currents were activated by 100 ms voltage steps (range − 100 to + 50 mV) in 10 mV increments from a holding potential of 0 mV. Control currents were recorded 1 and 5 min after establishment of whole-cell recording (WCR). PNU-99963 (1 μM) was subsequently applied for 2 min followed by 10 mM $BaCl_2$. (b) $I-V$ relationships of the currents generated by Kir6.2/SUR1 and Kir6.1/SUR2B from experiments shown above.

inhibition by PNU-99963 than observed with the other three clones ($P<0.05$). The relation between PNU-99963 concentration and current inhibition is shown in Figure 3. PNU-99963 inhibited all the four cloned channels in a concentration-dependent manner; the concentration at which half-maximum current was inhibited (IC_{50}) being 66.1 nM (slope factor 1.3), 41.4 nM (slope factor 1.3), 42.6 nM (slope factor 1.2) and 11.5 nM (slope factor 0.9) for Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B, respectively.

Effects of PNU-37883A on cloned channels were examined under similar conditions. Figure 4 shows that application of 30 μM PNU-37883A inhibited all four types of cloned currents, although to different extents. The mean inhibition by 30 μM PNU-37883A of Kir6.2/SUR1 current (measured at -100 mV and calculated as percentage block of 10 mM $BaCl_2$-sensitive current) was $13.7\pm4.9\%$ ($n=8$), of Kir6.2/SUR2A was $32.5\pm4.9\%$ ($n=6$), of Kir6.2/SUR2B was $52.3\pm4.6\%$ ($n=6$) and of Kir6.1/SUR2B was $52.4\pm9.0\%$ ($n=8$). The $I-V$ relationships of Kir6.2/SUR1 and Kir6.1/SUR2B currents measured during the last 10 ms of the voltage steps are shown in Figure 4b. The block by PNU-37883A was voltage-independent, giving a linear $I-V$ and similar percentage inhibition of the control K_{ATP} current at all potentials studied (-100 to 50 mV). The selective inhibition by PNU-37883A on Kir6.2/SUR2B and Kir6.1/SUR2B was further examined as shown in Figure 5. It can be seen that PNU-37883A caused a concentration-dependent inhibition of Kir6.2/SUR2B and Kir6.1/SUR2B currents with IC_{50} of 15.2 μM (slope factor 0.5) and 6 μM (slope factor 0.9), respectively. In contrast, PNU-37883A at 100 μM caused a small although significant inhibition of Kir6.2/SUR1 and Kir6.2/SUR2A currents, blocking the current less than 40% at this concentration.

A C-terminal truncated pore-forming subunit of Kir6.2 (Kir6.2Δ26), lacking the last 26 amino acids, is capable of forming a functional channel in the absence of SUR (Tucker *et al*., 1997). This has proved to be a useful tool for discriminating the site of action of various agents on K_{ATP} channels. Figure 6 shows that PNU-99963 at concentration up to 3 μM had no significant effect on Kir6.2Δ26 current, whereas this was sufficient to almost fully block the current carried by Kir6.2/SUR or Kir6.1/SUR2B. At higher concentrations, PNU-99963 did inhibit the current, although the concentration required for comparable inhibition was thousand times higher than that required for Kir6.2/SUR current. Thus, the mean inhibition of Kir6.2Δ26 current by PNU-99963 at 30 μM was $\sim$50%, which contrasts an IC_{50} of $\sim$50 nM for PNU-99963 inhibition of Kir6.2/SUR currents.

Effects of PNU-37883A on Kir6.2Δ26 current were also examined. Figure 7a shows that PNU-37883A at 30 μM inhibited Kir6.2Δ26 current on average by 90% with $BaCl_2$ (10 mM) causing a slightly greater inhibition of the current. Figure 7c shows that inhibition by PNU-37883A was concentration-dependent with IC_{50} of 4.6 μM (slope factor 1.0). The IC_{50} was lower (15 μM) when Kir6.2 was coexpressed with SUR. Therefore, the effects of PNU-37883A on Kir6.2Δ26 current were further examined in the presence of SUR2B. Figure 7b and d shows the current traces and the corresponding concentration – response curve of such experiments. Similar to the observation when Kir6.2/SUR2B was coexpressed, application of PNU-37883A led to a concentration-dependent inhibition of Kir6.2Δ26/SUR2B current with IC_{50} of 38 μM and a lower slope factor (0.4).

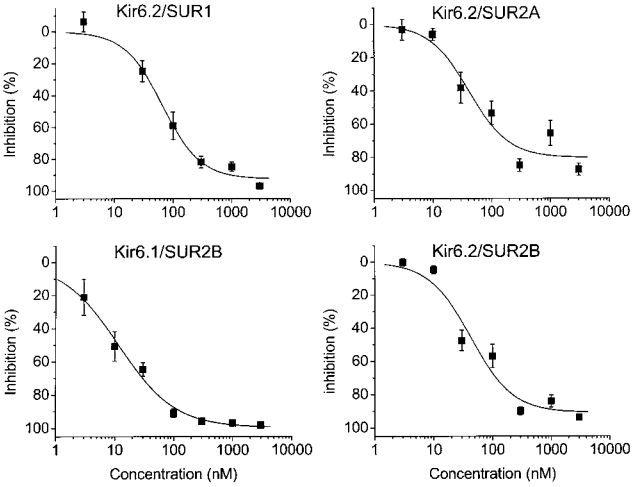

Figure 3 Concentration – response relationship for PNU-99963 of Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B currents stably expressed in HEK293 cells. Inhibition by PNU-99963 was calculated as the percentage inhibition of 10 mM $BaCl_2$-sensitive current. Data are mean $\pm$ s.e.m., $n=4-12$.

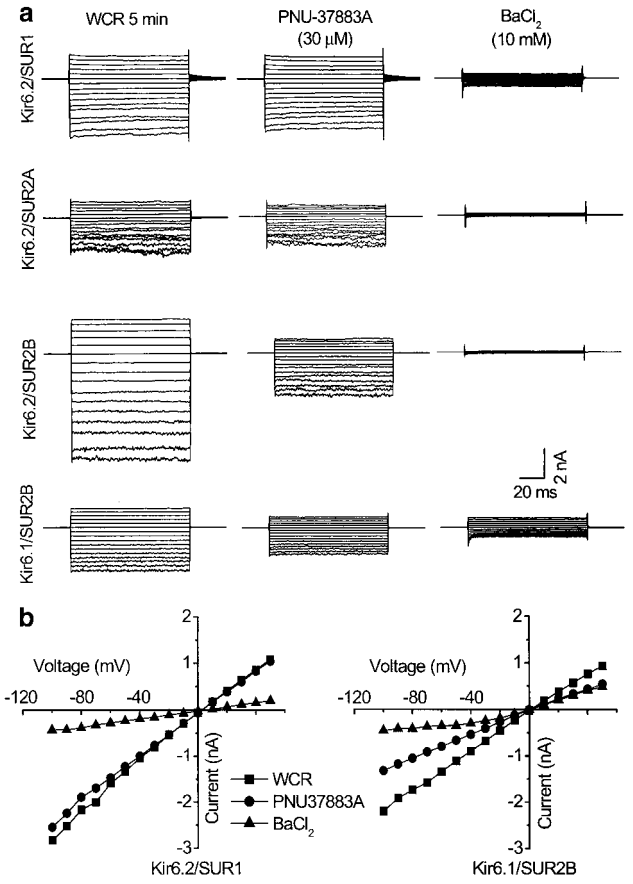

Figure 4 Effects of PNU-37883A on Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B currents stably expressed in HEK-293 cells. (a) Whole-cell currents were activated by 100 ms voltage steps in 10 mV increments from a holding potential of 0 mV. Control shows the currents recorded at 5 min after establishment of WCR. PNU-37883A (30 μM) was applied for at least 2 min followed by 10 mM $BaCl_2$. (b) The $I-V$ relationships of Kir6.2/SUR1 and Kir6.1/SUR2B.

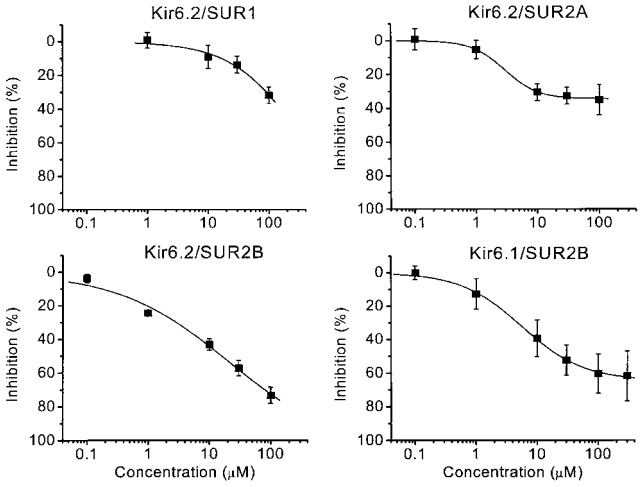

Figure 5 Concentration – response relationship for PNU-37883A of Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B currents stably expressed in HEK-293 cells. Inhibition by PNU-37883A was calculated as the percentage inhibition of 10 mM $BaCl_2$-sensitive current. Data are mean ± s.e.m., $n = 4 – 10$.

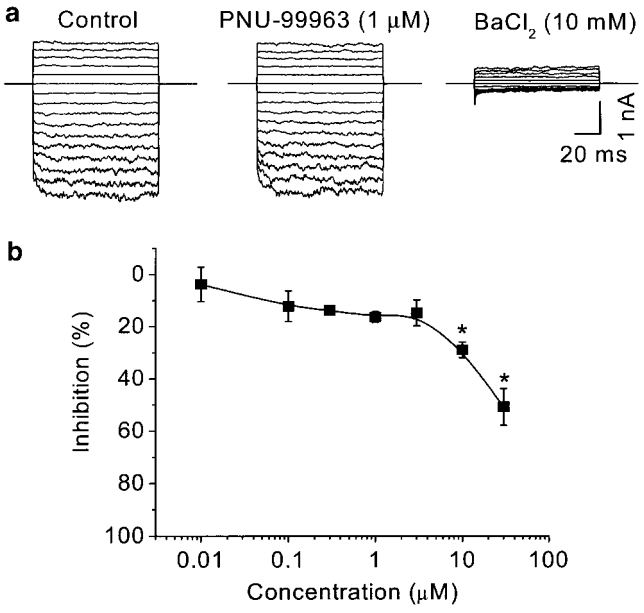

Figure 6 Effects of PNU-99963 on currents generated by transiently expressing Kir6.2Δ26 in HEK-293 cells. (a) Whole-cell currents were activated by 100 ms voltage steps in 10 mV increments from a holding potential of 0 mV. Current was recorded within 5 min after the establishment of WCR (control), after 1 μM PNU-99963 had been applied for 2 min and after 10 mM $BaCl_2$. (b) Concentration – response relationship for PNU-99963 on Kir6.2Δ26 current transiently expressed in HEK-293 cells. Inhibition by PNU-99963 was calculated as the percentage inhibition of 10 mM $BaCl_2$-sensitive current. $^*P < 0.05$ when compared with 10 nM PNU-99963. Data are mean ± s.e.m., $n = 4 – 11$.

Discussion

K_{ATP} channel modulators have diverse specificity towards K_{ATP} channels by acting on the different subunits of the channel. In the present study, we examined the effects of the nonsulphonylurea agents, PNU-99963 and PNU-37883A, on four types of cloned K_{ATP} channels corresponding to the

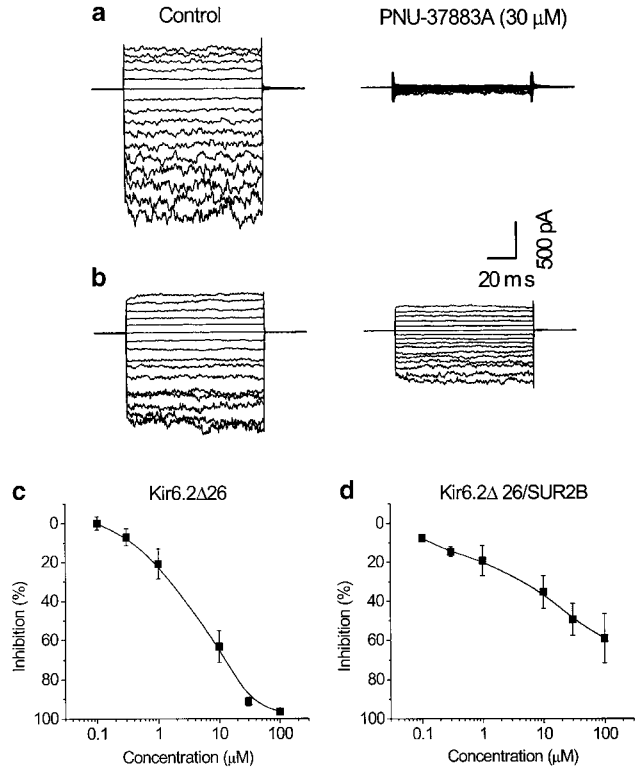

Figure 7 Effects of PNU-37883A on currents generated by transiently expressing Kir6.2Δ26 alone (a) or with SUR2B (b) in HEK-293 cells. Whole-cell currents were activated by 100 ms voltage steps in 10 mV increments from a holding potential of 0 mV. Currents were recorded at 5 min after establishment of WCR (control) and following the application of 30 μM PNU-37883A for at least 2 min. Concentration – response relationship for PNU-37883A on currents generated by Kir6.2Δ26 (c) and Kir6.2Δ26/SUR2B (d). Inhibition by PNU-37883A was calculated as the percentage inhibition of 10 mM $BaCl_2$-sensitive current. Data are mean ± s.e.m., $n = 4 – 6$.

pancreatic β cell, cardiac and smooth muscle K_{ATP} channels. Our results demonstrate that PNU-99963 and PNU-37883A, two structurally different compounds, inhibit K_{ATP} channels by acting on different sites, the former by interacting with a high affinity site located on SUR and the latter by interacting with the pore-forming subunits, Kir6.1 or Kir6.2.

PNU-99963 potently inhibited all the four cloned K_{ATP} channels with an IC_{50} in the low nanomolar range. Thus, this compound represents the most potent K_{ATP} inhibitor known for cardiac and smooth muscle K_{ATP} channels, and unlike glibenclamide, does not discriminate between SUR1 and SUR2. This is not surprising given that PNU-99963 is a derivative of pinacidil, and pinacidil has been shown to activate all the four cloned K_{ATP} channels (Liu *et al.*, 2001). It is generally accepted that pinacidil activates K_{ATP} channels via the SUR subunit, although the exact binding site has not been determined (Ashcroft & Gribble, 2000; Gribble *et al.*, 2000). Our results also provide strong evidence that PNU-99963 acts on the SUR subunit, and it is tempting to speculate that PNU-99963 shares the same binding site as pinacidil. This is supported by the observation that PNU-99963 up to 3 μM had no significant effects on the current generated by expressing Kir6.2Δ26 alone. PNU-99963 did inhibit Kir6.2Δ26 current at higher concentrations ($> 10\,\mu$M), suggesting that there might be a lower affinity binding site on Kir6.2, as there are with some sulphonylurea inhibitors (Bryan & Aguilar-

Bryan, 1999). However, it is unlikely that binding to this site contributes significantly to the inhibitory effect of PNU-99963, since full inhibition of Kir6.0/SUR occurred at a concentration of $\sim 1-3\,\mu$M. Given that the IC_{50} for inhibiting current (12–43 nM) or blood vessel relaxation induced by KCOs (18 nM) (Khan *et al*., 1997) is comparable, inhibiting K_{ATP} channels via the SUR subunit is likely to be the major mechanism underlying the inhibitory effects of PNU-99963 in smooth muscle.

Although PNU-99963 potently inhibited all the four cloned K_{ATP} channels, it was approximately four-fold more potent at inhibiting Kir6.1/SUR2B current compared with currents generated by the other three cloned K_{ATP} channels sharing the same pore-forming subunit, Kir6.2. Thus, different pore-forming subunits may have subtle effects on the inhibitory action of PNU-99963. It is likely that coexpression of Kir6.1 with SUR2B enhances SUR2B affinity for PNU-99963 via interaction between these two types of subunits. It has been indeed demonstrated that the SUR2B expressed with Kir6.1 has a higher affinity for glibenclamide than SUR2B alone; the equilibrium dissociation constant of glibenclamide binding to SUR was changed from 32 to 6 nM when coexpressed with Kir6.1 (Russ *et al*., 1999). The enhancement of ligand binding to one subunit by coexpression of another subunit is also seen where potency of ATP in blocking the Kir6.2Δ26 channel was increased by three-fold after coexpression with SUR1 (Tucker *et al*., 1997).

Early studies with PNU-37883A showed that in isolated mesenteric arteries from various animal origins, it antagonised relaxation induced by diverse K_{ATP} channel openers, such as cromakalim, pinacidil and minoxidil sulphate with IC_{50} of 1 μM (Meisheri *et al*., 1993b). More direct evidence regarding the selectivity of this agent has come from recent electrophysiological experiments in native myocytes (Wellman *et al.,* 1999). In these studies, PNU-37883A significantly inhibited K_{ATP} currents recorded from isolated mesenteric artery smooth muscle cells with an IC_{50} of 3.5 μM, with little effect on K_{ATP} currents recorded from cardiac and skeletal muscle cells although higher concentrations (100 μM) were found to produce modest inhibition (25–38%). This is in broad agreement with our results in the various K_{ATP} channel clones. We found that PNU-37883A at a concentration of 30 μM inhibited basal current by $\sim$52% in smooth muscle K_{ATP} clones (Kir6.2/SUR2B and Kir6.1/SUR2B), whereas currents generated from the cardiac (Kir6.2/SUR2A) or β cell (Kir6.2/SUR1) clones were only inhibited by $\sim 14-33\%$ at the same concentration.

In the present study, we compared the effects of PNU-37883A on all the four cloned K_{ATP} channels, Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.1/SUR2B. Consistent with experiments in native tissues, we found that PNU-37883A had a degree of vascular selectivity, although our results show that PNU-37883A could not distinguish between smooth muscle subtypes. This is different in some respects to previous reported experiments, where PNU-37883A inhibited the Kir6.1/SUR1 and Kir6.1/SUR2B currents expressed in *Xenopus laevis* oocytes with an IC_{50} of 32 and 3.5 μM respectively, but had little effect on Kir6.2/SUR1 and Kir6.2/SUR2B currents (Surah-Narwal *et al*., 1999; Kovalev *et al.*, 2001). The reasons for these discrepancies are not known, but a number of factors should be taken into account. Firstly, the expression systems used in these experiments are different, that is, *Xenopus laevis oocytes* vs mammalian cells (HEK-293 cell line). Secondly, we dialysed cells with low ATP solution to release the inhibition of channel by endogenous ATP, whereas others have used either diazoxide or pinacidil to activate currents. In the latter, the intracellular concentration of nucleotide was unknown. A considerable number of experiments have shown that effects of both K_{ATP} openers and blockers are modulated by the intracellular nucleotides (Jahangir *et al*., 1994; Tucker *et al*., 1997; D'hahan *et al*., 1999; Ashcroft & Gribble, 2000; Gribble *et al*., 2000).

Early experiments showed that glibenclamide and PNU-37883A potentiated each other's inhibitory effects on relaxation induced by KCOs in isolated rabbit mesenteric artery. This synergistic interaction indicated different sites of action for glibenclamide and PNU-37883A (Ohrnberger *et al*., 1993). Our experiments with Kir6.2Δ26 further identify that PNU-37883A acts on the pore-forming subunit, Kir6.2. How then can we explain that a pore blocker inhibits Kir6.2/SUR1 and Kir6.2/SUR2A currents to a much lesser degree than it does Kir6.2/SUR2B current, when all the three channels have the same pore-forming subunit? The mostly likely interpretation of our data is that coassembly of pore-forming subunit with SUR receptor affects drug binding to the pore. As discussed earlier, interaction between the pore and SUR receptor does play an important role in determining the pharmacological actions of K_{ATP} channel agents. Our results that the potency of the drug was seven-fold greater on Kir6.2Δ26 current than Kir6.2Δ26/SUR2B current support the idea that the SUR can modulate potency.

In conclusion, two structurally different nonsulphonylurea compounds, PNU-99963 and PNU-37883A, inhibit K_{ATP} channels at different molecular sites. The former potently inhibits these channels via the SUR and has little selectivity between the smooth muscle, cardiac or pancreatic β cell K_{ATP} channel clones. In contrast, PNU-37883A mediates its inhibitory effects through the pore-forming subunit, although the potency of inhibition is reduced by the presence of the SUR and influenced by the SUR subtype. While PNU-37883A has a degree of selectivity towards smooth muscle K_{ATP} channels, this compound does not appear to discriminate between the two subtypes.

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References

AGUILAR-BRYAN, L., CLEMENT, J.P., GONZALEZ, G., KUNJILWAR, K., BABENKO, A. & BRYAN, J. (1998). Toward understanding the assembly and structure of K_{ATP} channels. *Physiol. Rev.,* **78,** 227–245.

ASHCROFT, F.M. (1988). Adenosine 5′-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.,* **11,** 97–118.

ASHCROFT, F.M. & GRIBBLE, F.M. (2000). New windows on the mechanism of action of K_{ATP} channel openers. *Trends Pharmacol. Sci.,* **21,** 439–445.

BRYAN, J. & AGUILAR-BRYAN, L. (1999). Sulfonylurea receptors: ABC transporters that regulate ATP-sensitive K^+ channels. *Biochim. Biophys. Acta,* **1461,** 285–305.

CUI, Y., GIBLIN, J.P., CLAPP, L.H. & TINKER, A. (2001). A mechanism for ATP-sensitive potassium channel diversity: functional coassembly of two pore forming subunits. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 729 – 734.

CUI, Y., TINKER, A. & CLAPP, L.H. (2002a). Potent inhibition of cloned K_{ATP} channels stably expressed in human embryonic kidney (HEK) 293 cells by the pinacidil derivative, PNU-99963. *Br. J. Pharmacol.,* **135,** 118P (Abstract).

CUI, Y., TRAN, S., TINKER, A. & CLAPP, L.H. (2002b). The molecular composition of K_{ATP} channels in human pulmonary artery smooth muscle cells and their modulation by growth. *Am. J. Respir. Cell Mol. Biol.,* **26,** 135 – 143.

D'HAHAN, N., PROST, A., MOREAU, C.L., JACQUET, H., ALEKSEEV, A.E., TERZIC, A. & VIVAUDOU, M. (1999). Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 12162 – 12167.

GIBLIN, J.P., CUI, Y., CLAPP, L.H. & TINKER, A. (2002). Assembly limits the pharmacological complexity of ATP-sensitive potassium channels. *J. Biol. Chem.,* **277,** 13717 – 13723.

GIBLIN, J.P., LEANEY, J.L. & TINKER, A. (1999). The molecular assembly of ATP-sensitive potassium channels: determinants on the pore forming subunit. *J. Biol. Chem.,* **274,** 22652 – 22659.

GRIBBLE, F.M., REIMANN, F., ASHFIELD, R. & ASHCROFT, F.M. (2000). Nucleotide modulation of pinacidil stimulation of the cloned K_{ATP} channel Kir6.2/SUR2A. *Mol. Pharmacol.,* **57,** 1256 – 1261.

GRIBBLE, F.M., TUCKER, S.J., SEINO, S. & ASHCROFT, F.M. (1998). Tissue specificity of sulfonylureas: studies on cloned cardiac and beta-cell K_{ATP} channels. *Diabetes,* **47,** 1412 – 1418.

HUMPHREY, S.J., SMITH, M.P., CIMINI, M.G., BUCHANAN, L.V., GIBSON, J.K., KHAN, S.A. & MEISHERI, K.D. (1996). Cardiovascular effects of the K-ATP channel blocker U-37883A and structurally related morpholinoguanidines. *Meth. Find. Exp. Clin. Pharmacol.,* **18,** 247 – 260.

INAGAKI, N., GONOI, T., CLEMENT, J.P., NAMBA, N., INAZAWA, J., GONZALEZ, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of $I_{K_{ATP}}$: an inward rectifier subunit plus the sulfonylurea receptor. *Science,* **270,** 1166 – 1170.

INAGAKI, N., GONOI, T., CLEMENT, J.P., WANG, C.Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron,* **16,** 1011 – 1017.

ISOMOTO, S., KONDO, C., YAMADA, M., MATSUMOTO, S., HIGASHIGUCHI, O., HORIO, Y., MATSUZAWA, Y. & KURACHI, Y. (1996). A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J. Biol. Chem.,* **271,** 24321 – 24324.

JAHANGIR, A., TERZIC, A. & KURACHI, Y. (1994). Intracellular acidification and ADP enhance nicorandil induction of ATP sensitive potassium channel current in cardiomyocytes. *Cardiovasc. Res.,* **28,** 831 – 835.

KHAN, S.A., HIGDON, N.R., HESTER, J.B. & MEISHERI, K.D. (1997). Pharmacological characterization of novel cyanoguanidines as vascular K_{ATP} channel blockers. *J. Pharmacol. Exp. Ther.,* **283,** 1207 – 1213.

KOH, S.D., BRADLEY, K.K., RAE, M.G., KEEF, K.D., HOROWITZ, B. & SANDERS, K.S. (1998). Basal activation of ATP-sensitive potassium channels in murine colonic smooth muscle cell. *Biophys. J.*, **75,** 1793 – 1800.

KOVALEV, H., LODWICK, D. & QUAYLE, J.M. (2001). Inhibition of cloned K_{ATP} channels by the morpholinoguanidine PNU-37883A. *J. Physiol. (London),* **531P,** P89.

LIU, Y., REN, G., O'ROURKE, B., MARBAN, E. & SEHARASEYON, J. (2001). Pharmacological comparison of native mitochondrial K_{ATP} channels with molecularly defined surface K_{ATP} channels. *Mol. Pharmacol.,* **59,** 225 – 230.

MEISHERI, K.D., HUMPHREY, S.J., KHAN, S.A., CIPKUSDUBRAY, L.A., SMITH, M.P. & JONES, A.W. (1993a). 4-Morpholinecarboximidine-*n*-1-adamantyl-*n*′-cyclohexylhydrochloride (U-37883A): pharmacological characterization of a novel antagonist of vascular ATP- sensitive K^+ channel openers. *J. Pharmacol. Exp. Ther.,* **266,** 655 – 665.

MEISHERI, K.D., KHAN, S.A. & MARTIN, J.L. (1993b). Vascular pharmacology of ATP-sensitive K^+ channels – interactions between glyburide and K^+ channel openers. *J. Vasc. Res.,* **30,** 2 – 12.

O'BRIEN, A.J., THAKUR, G., CUI, Y., SINGER, M. & CLAPP, L.H. (2002). Inhibitors of the pore-forming subunit of the K_{ATP} channel partially reverse endotoxin-induced vascular hyporeactivity in rat superior mesenteric artery. *Br. J. Pharmacol.,* **135,** 116P (Abstract).

OHRNBERGER, C.E., KHAN, S.A. & MEISHERI, K.D. (1993). Synergistic effects of glyburide and U-37883A, two structurally different vascular ATP-sensitive potassium channel antagonists. *J. Pharmacol. Exp. Ther.,* **267,** 25 – 30.

RUSS, U., HAMBROCK, A., ARTUNC, F., LOFFLER-WALZ, C., HORIO, Y., KURACHI, Y. & QUAST, U. (1999). Coexpression with the inward rectifier K^+ channel Kir6.1 increases the affinity of the vascular sulfonylurea receptor SUR2B for glibenclamide. *Mol. Pharmacol.,* **56,** 955 – 961.

SURAH-NARWAL, S., XU, S.Z., MCHUGH, D., MCDONALD, R.L., HOUGH, E., CHEONG, A., PARTRIDGE, C., SIVAPRASADARAO, A. & BEECH, D.J. (1999). Block of human aorta Kir6.1 by the vascular K_{ATP} channel inhibitor U37883A. *Br. J. Pharmacol.,* **128,** 667 – 672.

TUCKER, S.J., GRIBBLE, F.M., ZHAO, C., TRAPP, S. & ASHCROFT, F.M. (1997). Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature,* **387,** 179 – 183.

WELLMAN, G.C., BARRETT-JOLLEY, R., KOPPEL, H., EVERITT, D. & QUAYLE, J.M. (1999). Inhibition of vascular K_{ATP} channels by U-37883A: a comparison with cardiac and skeletal muscle. *Br. J. Pharmacol.,* **128,** 909 – 916.

YAMADA, M., ISOMOTO, S., MATSUMOTO, S., KONDO, C., SHINDO, T., HORIO, Y. & KURACHI, Y. (1997). Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive K^+ channel. *J. Physiol. (London),* **499,** 715 – 720.

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