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Inhibition of recombinant K_{ATP} channels by the antidiabetic agents midaglizole, LY397364 and LY389382

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Abstract

Most imidazolines inhibit ATP-sensitive K^+ (K_{ATP}) channels. Since these drugs are potentially clinically relevant insulin secretagogues, it is important to know whether extrapancreatic K_{ATP} channels are targeted. We examined the effects of three imidazoline-derived antidiabetic drugs on the cloned K_{ATP} channel, expressed in *Xenopus laevis* oocytes, and their specificity for interaction with the pore-forming Kir6.2 or the sulphonylurea receptor (SUR) 1 subunit. Midaglizole, LY397364 and LY389382 blocked Kir6.2ΔC currents with IC_{50} of 3.8, 6.1 and 0.7 μM, respectively. The block of Kir6.2/SUR1 currents by LY397364 and LY389382 was best fit by a two-site model, suggesting that these drugs also interact with SUR1. However, since all three drugs interact with the Kir6.2 subunit, and Kir6.2 forms the pore of extrapancreatic K_{ATP} channels, these drugs are unlikely to be specific for the β-cell.

Keywords: Midaglizole; K_{ATP} channel; Kir6.2; Imidazoline; Diabetes; Insulin secretion

1. Introduction

The ATP-sensitive K^+-channel (K_{ATP} channel) plays a key role in insulin secretion from pancreatic β-cells (Ashcroft and Rorsman, 1989). It is closed both by glucose metabolism and by sulphonylureas, which are used in the treatment of non-insulin-dependent diabetes mellitus. This produces a membrane depolarization, which activates voltage-dependent Ca^{2+}-channels, enhances Ca^{2+} influx, and thereby stimulates insulin release. K_{ATP} channels are also important for the physiology and pathophysiology of many other tissues (Ashcroft and Ashcroft, 1990; Trapp and Ashcroft, 1997; Ashcroft and Gribble, 1999). For example, they are involved in the response to cardiac and cerebral ischaemia, regulate vascular smooth muscle tone, modulate transmitter release at central synapses and mediate K^+ flux across epithelial cells (Nichols and Lederer, 1991; Davis et al., 1991; Quayle et al., 1997; Aguilar-Bryan and Bryan, 1999).

The β-cell K_{ATP} channel is an octameric complex of two different protein subunits: an inwardly rectifying K^+ channel, Kir6.2, and a sulphonylurea receptor, SUR1 (Sakura et al., 1995; Inagaki et al., 1995). The former acts as an ATP-sensitive K^+ channel pore, while SUR1 is a channel regulator, which endows Kir6.2 with sensitivity to drugs such as the inhibitory sulphonylureas and the K^+ channel opener diazoxide (Inagaki at al., 1995; Tucker et al., 1997). K_{ATP} channels in different tissues share a common Kir6.2 subunit, but possess different types of SUR subunit, which accounts for their different drug sensitivities (Ashcroft and Gribble, 1999). The β-cell K_{ATP} channel is composed of Kir6.2 and SUR1, the cardiac type of Kir6.2 and SUR2A and the smooth muscle type, probably, of Kir6.2 and SUR2B (Inagaki et al., 1995, 1996; Sakura et al., 1995; Isomoto et al., 1996). Although wild-type K_{ATP} channels require both types of subunit (Kir6.2 and SUR) for functional activity, a mutant form of Kir6.2 with a C-terminal truncation of 26 or 36 amino acids (Kir6.2ΔC) is capable of independent expression (Tucker et al., 1997). Kir6.2ΔC

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therefore provides a useful tool for studying the effects of drugs on the pore-forming subunit of the K_{ATP} channel.

It has been known for many years that certain drugs that contain an imidazoline moiety, including several classical α-adrenoreceptor antagonists, act as potent stimulators of insulin secretion (Plant and Henquin, 1990; Jonas et al., 1991; Chan et al., 1991). There is good evidence that the insulinotropic effects of these drugs do not result from antagonism of α-adrenoreceptors, but rather from inhibition of K_{ATP} channels in the β-cell plasma membrane (Plant and Henquin, 1990; Jonas et al., 1991; Chan et al., 1991; Dunne, 1991; Dunne et al., 1995). One of the most potent imidazolines is phentolamine, which blocks native K_{ATP} currents in β-cells half-maximally at 0.7 μM when added to the intracellular solution (Dunne, 1991). This inhibition is caused by direct interaction of phentolamine with the Kir6.2 subunit (Proks and Ashcroft, 1997). Because Kir6.2 serves as the pore-forming subunit for cardiac and smooth muscle, this finding may explain why imidazolines also have cardiovascular actions, which are independent of α-adrenoreceptor activation (Gould and Reddy, 1976; Lee et al., 1995).

Studies performed over the past few years have suggested that some imidazoline derivatives might stimulate insulin secretion in a more glucose-dependent fashion than sulphonylureas (Efanov et al., 2001b; Mest et al., 2001). Since medication-induced hypoglycemia is a major risk for diabetic patients, drugs that potentiate glucose-induced insulin secretion, but do not initiate insulin secretion under low blood glucose levels, are desired. The degree of glucose dependence of the insulinotropic action observed for these imidazoline-derived drugs varies, e.g. LY389382, which is used in this study, exhibits a stronger glucose dependence than midaglizole (Mest et al., 2001), and novel imidazoline-based compounds are synthesized to improve this property further.

Such compounds have also been shown to stimulate insulin secretion by a K_{ATP} channel-independent mechanism (Efanov et al., 2001a,b). The imidazoline RX871024 stimulated insulin secretion from islets from SUR1 deficient mice (Efanov et al., 2001a). However, RX871024 has also been shown to inhibit Kir6.2 currents directly (Efanov et al., 2001a), whereas a different imidazoline, BL11282, did not influence whole cell K_{ATP} currents in rat β-cells (Efanov et al., 2001b).

The aim of this study was to examine whether the novel imidazoline-derived compound LY397364 also stimulates insulin secretion in a highly glucose-dependent manner, and whether such compounds do or do not affect K_{ATP} channel (Kir6.2/SUR1) activity at concentrations that induce insulin secretion. In addition to LY389382 and LY397364, midaglizole was used because clinical data is available for this compound (Kawazu et al., 1987). To assess their possible tissue specificity, the effects of these drugs were studied additionally on the truncated Kir6.2 subunit expressed in the absence of the sulphonylurea receptor.

2. Materials and methods

2.1. Insulin secretion

MIN6 cells (passage numbers 28–32) were cultured and prepared for insulin secretion experiments as described previously (Mest et al., 2001). After starvation of the cells for 1 h in Earle's Balanced Salt Solution (EBSS, Sigma, Taufkirchen, Germany) plus 0.1% bovine serum albumine, the cells were incubated with various concentrations of LY397364 at 0 and 25 mM glucose for an additional hour. The reaction was terminated by filtration of the cell supernatant through Multiscreen MAHVN filterplates (Millipore, Eschborn, Germany). The filtered cell culture supernatant was stored at −20 °C until the insulin concentration was determined using an anti-rat insulin radioimmunoassay kit from Linco (Biotrend, Köln, Germany).

Islets of Langerhans were isolated from the pancreata of male Wistar rats (body weight of 200 to 250 g) as described previously (Mest et al., 2001). Insulin secretion studies were performed in static incubations. Islets were hand-picked into culture dishes and pre-incubated in EBSS containing 0.1% bovine serum albumine supplemented with 3.3 mM glucose for 30 min at 37 °C. Batches of five islets were hand picked into 24-well tissue culture plates and incubated for 90 min at 37 °C in EBSS (+0.1% bovine serum albumine) supplemented with 3.3 or 16.7 mM glucose and LY397364 added. Chilling of the test plates on ice terminated the incubations. The supernatant was removed and stored at −20 °C until it was assayed for insulin.

2.2. Molecular biology

Mouse Kir6.2 (Genbank D50581; Inagaki et al., 1995; Sakura et al., 1995) and rat SUR1 (Genbank L40624; Aguilar-Bryan et al., 1995) cDNAs were cloned in the pBF vector. A truncated form of Kir6.2 (Kir6.2ΔC), which lacks the C-terminal 36 amino acids and forms functional channels in the absence of SUR, was prepared as described previously (Tucker et al., 1997). Capped mRNA was synthesized using the mMESSAGE mMACHINE large scale in vitro transcription kit (Ambion, Austin, TX, USA), as previously described (Gribble et al., 1997).

Oocytes were harvested from female *Xenopus laevis* and manually defolliculated as described previously (Gribble et al., 1997). Individual oocytes were either injected with ~1 ng Kir6.2ΔC mRNA or coinjected with ~0.1 ng Kir6.2 mRNA and ~2 ng of mRNA encoding SUR1. The final injection volume was 50 nl/oocyte. Isolated oocytes were maintained in Barth's solution and studied 1–4 days after injection (Gribble et al., 1997).

2.3. Electrophysiology

Patch pipettes were pulled from thick-walled borosilicate glass and had resistances of 250–500 kΩ when filled with

pipette solution. Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV and at 20–24 °C. Currents were evoked by repetitive 3 s voltage ramps from − 110 to + 100 mV and recorded using either an EPC7 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) or an Axopatch 200B (Axon Instruments, Union City, USA).

The pipette (external) solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained (mM): 107 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.2 with KOH; final $[K^+]$ ~ 140 mM). Stock solutions of drugs were prepared at a concentration of 100 mM by dissolving them in DMSO (dimethylsulphoxide). Stock solutions were kept at 4 °C and experimental solutions were prepared freshly each day. Rapid exchange of solutions was achieved by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the bath. Test solutions were applied in random order. Patches were exposed to 1 mM MgATP at intervals throughout the experiment to reverse channel rundown.

Midaglizole (developed by Daiichi Seiyaku), LY397364 and LY389382 (Fig. 1) were synthesized at Lilly Forschung (Hamburg, Germany). All other chemicals were from Sigma.

2.4. Data analysis

The slope conductance was measured by fitting a straight line to the current–voltage relation between − 20 and − 100 mV: the mean conductance from five consecutive ramps was calculated in each solution.

2.4.1. Analysis of midaglizole

Dose–response curves were constructed by expressing the conductance in the presence of drug as a fraction of the mean of the conductances measured in control solution before and after addition of the drug. Concentration–response curves for Kir6.2/SUR1 currents were fit to the Hill equation:

$$\frac{G}{G_c} = \frac{(1-B)}{(1+([X]/IC_{50})^h)} + B \quad (1)$$

where G is the conductance in the presence of the drug, G_c is the conductance in control solution, $[X]$ is the drug concentration, IC_{50} is the drug concentration at which inhibition is half maximal, h is the Hill coefficient (slope factor) and B is the fraction of the current remaining when inhibition is maximal. Fits were obtained using Microcal Origin software and data is presented as mean ± 1 S.E.M.

2.4.2. Analysis of LY397364 and LY389382

Inhibition of Kir6.2/SUR1 currents by LY397364 and LY389382 was slow and only poorly reversible. This meant that at low drug concentrations, it was difficult to distinguish a blocking action of the drug from channel rundown

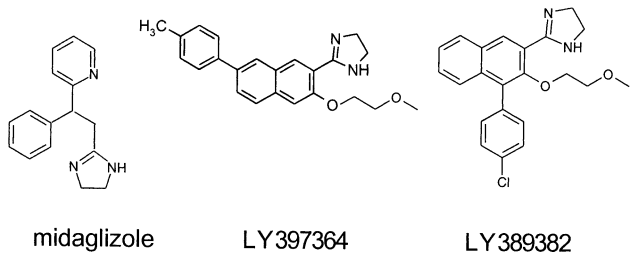

Fig. 1. Structures of midaglizole, LY389382 and LY397364. The structure of LY389382 and LY397364 is based on the same naphthyl platform. Midaglizole, which was developed by Daiichi Seiyaku, has a different basis. All three compounds were synthesized by Lilly Forschung. The molecular weight of the compounds is as follows: midaglizole, 251.33; LY397364, 360.46 and LY389382, 380.88.

because both occurred on the same time scale. Therefore, the following strategy was used to construct dose–inhibition curves. First, drug concentrations were applied in ascending order. Secondly, we controlled for channel rundown. Rundown was well fit by the exponential function:

$$R(t) = A + Be^{(-t/\tau)} \quad (2)$$

where $R(t)$ is the amplitude of the conductance at time t, τ is the time constant of rundown and A and B are constants. This function was fit to the conductance values obtained in control solution at the start of the experiment, extrapolated throughout the rest of the experiment and compared to the actual conductance measured in control solution. If the measured value agreed with that predicted from Eq. (2), then the drug was taken to be reversible, but if they were not in agreement, the drug was considered to block irreversibly. The extent of inhibition was calculated in different ways, depending on whether the block was reversible or irreversible.

If the block was reversible, and the time-course of block was fast, the extent of block was determined as the conductance in the presence of the drug divided by the mean of the conductances obtained in the control solutions before and after drug application. If the block was irreversible, the conductance in the test solution was expressed as a fraction of that predicted in control solution at the time of drug application from the exponential rundown function.

A further complication was that in some patches, the time-course of block was slow and it was not possible to measure the steady-state conductance in the presence of the drug accurately. In this case, the steady-state block was estimated as:

$$B_{ss} = CR(t=0)/G(t=0) \quad (3)$$

where B_{ss} is the steady-state block in the presence of the drug, $R(t=0)$ is the conductance at the time of drug

$$G(t)/R(t) = C + D\exp(-t/T) \quad (4)$$

application estimated from Eq. (2) and $G(t=0)$ is the measured conductance at the time of drug application. The parameter C was obtained by fitting an exponential function to the ratio

where $G(t)$ is the conductance measured at time t in the presence of the drug, $R(t)$ is the conductance at time t in control solution predicted from Eq. (2) and C, D and T are constants. $G(t)$ and $R(t)$ were obtained for all data points throughout the time of drug application. This method ensures that the time course of the block is corrected for rundown.

Concentration–response curves for Kir6.2ΔC currents were fit to Eq. (1). Concentration–response curves for Kir6.2/SUR1 were fit to the following equation (Gribble et al., 1997):

$$\frac{G}{G_c} = xy \tag{5}$$

where G is the conductance in the presence of the drug, G_c is the conductance in control solution, x is a term describing the high affinity site and y is a term describing the low affinity site.

$$x = L + \frac{(1-L)}{(1+([X]/\mathrm{IC}_{50_1})^{h1})} \tag{6}$$

$$y = \frac{1}{(1+([X]/\mathrm{IC}_{50_2})^{h2})} \tag{7}$$

where $[X]$ is the drug concentration, IC_{50_1} and IC_{50_2} are the drug concentrations at which inhibition is half maximal at high and low-affinity sites, respectively; $h1$, $h2$ are the Hill coefficients (slope factors) for the high- and low-affinity sites, respectively; and L is the fractional conductance remaining when the high-affinity sites are maximally occupied.

3. Results

3.1. Stimulation of insulin secretion by LY397364

LY397364 stimulates insulin secretion from rat pancreatic islets in a glucose-dependent manner (Fig. 2). In the presence of 16.7 mM glucose, insulin secretion is increased from 4 ng/ml in the absence of LY397364 to 11 ng/ml at saturating concentrations of LY397364. Half-maximal stimulation of insulin release was observed at 105 ± 18 nM ($n=3$). In contrast, no stimulation of insulin secretion by LY397364 was observed in the presence of 3.3 mM glucose ($n=3$). Equivalent results were obtained from experiments with the mouse insulinoma cell line MIN6. At 25 mM glucose, insulin secretion was stimulated by LY397364 with an EC_{50} of 300 ± 80 nM ($n=3$), whereas no stimulation of secretion was observed in the absence of glucose.

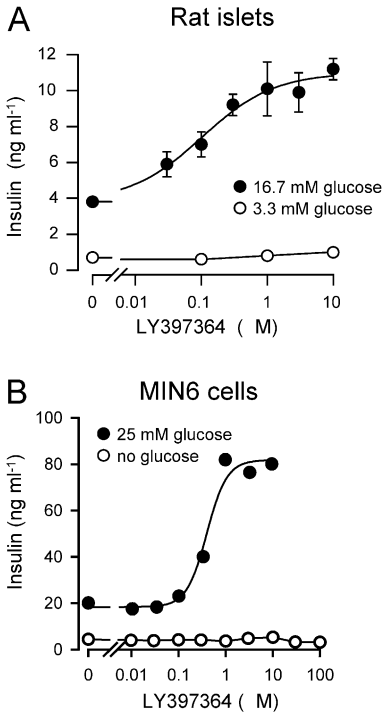

Fig. 2. Stimulation of glucose-dependent insulin release by LY397364. (A) Concentration-dependent effects of LY397364 on insulin secretion in isolated rat islets. The islets were incubated with LY397364 in the presence of 3.3 mM (○) and 16.7 mM (●) glucose. (B) Concentration-dependent effects of LY397364 on insulin secretion in MIN6 cells. The cells were incubated with LY397364 at 0 mM glucose (○) or 25 mM glucose (●). Data are mean values of three independent samples from a representative experiment. A nonlinear least squares fitting procedure using a four-parameter logistic equation was applied to calculate the curve representing the results at 16.7 and 25 mM glucose. Error bars represent one S.E.M. and were displayed when larger than the symbol.

3.2. Studies on recombinant K_{ATP} channels

We next explored whether LY397364 or other imidazoline-derived insulin secretagogues have direct inhibitory effects on recombinant K_{ATP} channels. Macroscopic currents were recorded in inside-out membrane patches from *Xenopus* oocytes coexpressing Kir6.2 and SUR1, or expressing Kir6.2ΔC in the absence of SUR. In all cases, the currents were small in the cell-attached configuration, but increased markedly when the patch was excised into nucleotide-free solution. This is consistent with the idea that the K_{ATP} channel is blocked in the intact oocyte by cytoplasmic nucleotides such as ATP.

3.2.1. Effects of midaglizole on recombinant K_{ATP} channels

Fig. 3 shows that application of 3 μM midaglizole to the intracellular membrane surface rapidly blocked Kir6.2/SUR1 currents and Kir6.2ΔC currents by 47 ± 2% ($n=7$)

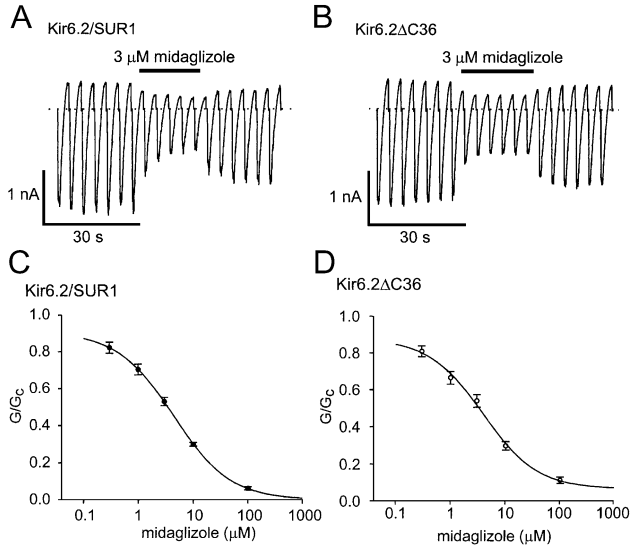

Fig. 3. Inhibition of Kir6.2/SUR1 and Kir6.2ΔC currents by midaglizole. (A–B) Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from −110 to +100 mV from oocytes coexpressing Kir6.2 and SUR1 or expressing Kir6.2ΔC. Midaglizole (3 μM) was added as indicated by the bars. Midaglizole concentration–response relationships measured for (C) Kir6.2/SUR1 or (D) Kir6.2ΔC currents. The macroscopic conductance in the presence of midaglizole (G) is expressed as a fraction of its mean amplitude in the absence of the drug (G_c). The symbols represent the mean, and the vertical bars indicate one S.E.M. Kir6.2/SUR1 data ($n=7$) were fit by Eq. (1) with $IC_{50}=4.4$ μM, $h=0.8$. Kir6.2ΔC data ($n=6$) were fit by Eq. (1) with $IC_{50}=3.8$ μM, $h=0.9$.

and 46 ± 3% ($n=6$), respectively. The relationship between midaglizole concentration and inhibition of Kir6.2/SUR1 and Kir6.2ΔC currents are shown in Fig. 3C and D. In both cases, the concentration–response curve is best described by assuming that the drug interacts with only a single site. Half-maximal inhibition (IC_{50}) of Kir6.2/SUR1 currents was produced by 4.4 ± 0.1 μM of the drug ($n=7$). A similar value was obtained for inhibition of Kir6.2ΔC currents: $IC_{50}=3.8\pm1.2$ μM ($n=6$). Thus, this result suggests that inhibition of the β-cell K_{ATP} channel by midaglizole is mediated by interaction of the drug with the Kir6.2 subunit. The Hill coefficients were 0.8 ± 0.1 ($n=7$) and 0.9 ± 0.3 ($n=6$) for Kir6.2/SUR1 and Kir6.2ΔC, respectively. This implies that binding of a single molecule of midaglizole is sufficient to block either the Kir6.2/SUR1 or Kir6.2ΔC channel.

3.2.2. Effects of LY397364 on recombinant K_{ATP} channels

LY397364, 10 μM, blocked both Kir6.2/SUR1 currents and Kir6.2ΔC currents by 84 ± 10% ($n=5$) and 67 ± 13% ($n=6$), respectively (Fig. 4A and B). In general, the block of Kir6.2ΔC currents by LY397364 was slow at drug concentrations of 10 μM and below. Inhibition of Kir6.2/SUR1 was more complex. At very low concentrations, the drug either had little effect (Fig. 4C) or induced a fast irreversible inhibition (Fig. 4D). At higher drug concentrations (from 1 to 10 μM), the block was slow and largely reversible, as in the case of Kir6.2ΔC.

The relationships between LY397364 concentration and the inhibition of Kir6.2ΔC and Kir6.2/SUR1 currents are shown in Fig. 4E and F. The concentration–response curve for Kir6.2ΔC currents was fit with Eq. (1) with an IC_{50} of 6.1 ± 0.3 μM ($n=5$), a Hill coefficient of 1.9 ± 0.1 (Fig. 4E) and $B=0$. In contrast, the block of Kir6.2/SUR1 currents was best described by assuming that the drug interacted with two sites (Fig. 4F; see Materials and methods for discussion of analysis). The high-affinity site had an IC_{50} of 3.8 ± 2.3 nM and the low-affinity site had an IC_{50} of 0.88 ± 0.55 μM ($n=5-8$). The Hill coefficients were 1.6 ± 0.7 and 0.7 ± 0.2 ($n=5-8$) for the high- and low-affinity sites, respectively. High-affinity inhibition produced a maximum block of 26 ± 5%. At these low drug concentrations, the

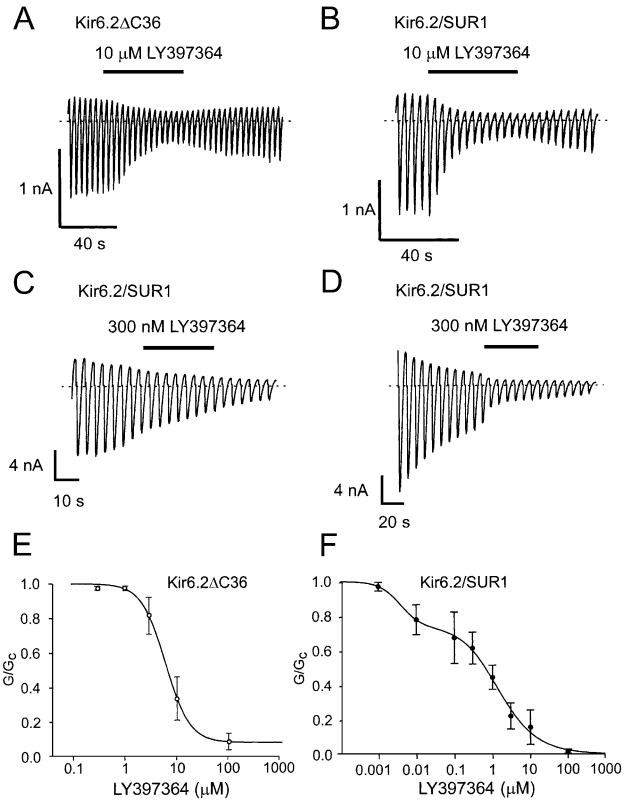

Fig. 4. Inhibition of Kir6.2/SUR1 and Kir6.2ΔC currents by LY397364. Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from −110 to +100 mV from oocytes expressing Kir6.2ΔC (A) or coexpressing Kir6.2 and SUR1 (B). LY397364 (10 μM) was added as indicated by the bars. (C–D) Inhibition of Kir6.2/SUR1 currents by 300 nM LY397364 was variable, ranging from no block to 40% inhibition. LY397364 was added as indicated by the bars. LY397364 concentration–response relationships measured for (E) Kir6.2ΔC or (F) Kir6.2/SUR1 currents. The macroscopic conductance in the presence of LY397364 (G) is expressed as a fraction of its mean amplitude in the absence of the drug (G_c). The symbols represent the mean, and the vertical bars indicate one S.E.M. Kir6.2ΔC data ($n=5$) were fit by Eq. (5) with $IC_{50}=6.1$ μM, $h=1.9$, $B=0.11$. Kir6.2/SUR1 data ($n=5-8$) were fit by Eq. (1) with $IC_{50_1}=3.8$ nM, $h=1.6$, $IC_{50_2}=0.88$ μM, $h=0.7$, $L=0.74$.

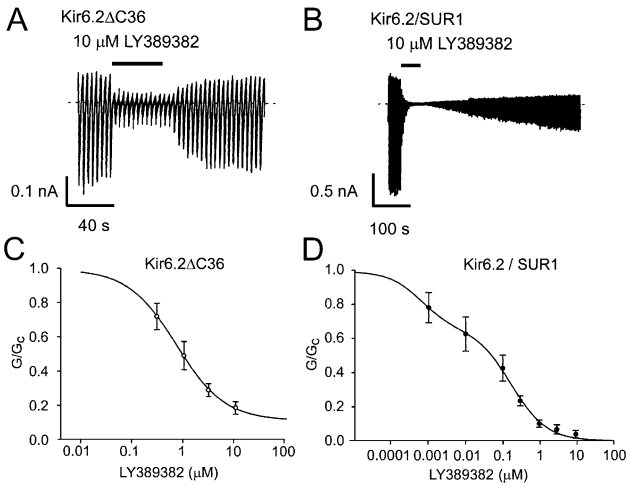

Fig. 5. Inhibition of Kir6.2/SUR1 and Kir6.2ΔC currents by LY389382. Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from −110 to +100 mV from oocytes expressing Kir6.2ΔC (A) or coexpressing Kir6.2 and SUR1 (B). LY389382 (10 μM) was added as indicated by the bars. LY389382 concentration–response relationships measured for Kir6.2ΔC (C) or Kir6.2/SUR1 (D) currents. The macroscopic conductance in the presence of LY389382 (G) is expressed as a fraction of its mean amplitude in the absence of the drug (G_c). The symbols represent the mean, and the vertical bars indicate one S.E.M. Kir6.2ΔC data ($n=5$) were fit by Eq. (1) with $IC_{50}=0.72$ μM, $h=0.93$, $B=0.11$. Kir6.2/SUR1 data ($n=5$–6) were fit by Eq. (5) with $IC_{50_1}=0.6$ nM, $h=0.89$, $IC_{50_2}=0.17$ μM, $h=0.89$, $L=0.67$.

amount of inhibition observed was rather variable; a drug concentration of 1–300 nM could produce anything from no effect to as much as 40% block.

3.2.3. Effects of LY389382 on recombinant K_{ATP} channels

Like the other drugs tested, LY389382 blocked both Kir6.2ΔC and Kir6.2/SUR1 currents (Fig. 5). The extent of block produced by 10 μM LY389382 was 82 ± 4% ($n=5$) for Kir6.2ΔC currents and 96 ± 2% ($n=5$) for Kir6.2/SUR1 currents. In general, the block of Kir6.2ΔC currents by LY389382 was slow and reversible at drug concentrations of 3 μM and below. As seen for LY397364, inhibition of Kir6.2/SUR1 currents was more complex. At very low concentrations, the drug either had little effect or induced a fast irreversible inhibition. At higher drug concentrations (from 1 to 3 μM), the block was slow and largely reversible, as in the case of Kir6.2ΔC currents. Above 3 μM, the block did not reverse completely.

Dose–response curves for inhibition of Kir6.2ΔC currents and Kir6.2/SUR1 currents by LY389382 are shown in Fig. 5C and D. As in the case of LY397364, the block of Kir6.2ΔC currents is best fit by a single-site model, while that of Kir6.2/SUR1 currents requires a two-site model. For Kir6.2ΔC currents, the IC_{50} was 0.72 ± 0.05 μM ($n=5$) and the Hill coefficient was 0.9 ± 0.1. For Kir6.2/SUR1 currents, the high-affinity site had an IC_{50} of 0.6 ± 0.3 nM and the low-affinity site had an IC_{50} of 0.17 ± 0.04 μM ($n=5$–6). The Hill coefficient for the low-affinity site was 0.9 ± 0.1; that of the high affinity was set to the same value. The maximum extent of block of the high-affinity inhibition was 33 ± 5%. However, as for LY397364, the amount of high-affinity inhibition varied considerably between individual patches.

4. Discussion

Our results demonstrate that all three compounds interact with the Kir6.2 subunit to produce channel inhibition. They also demonstrate that LY397364 stimulates insulin secretion from rat pancreatic islets and MIN6 cells in a highly glucose-dependent fashion like LY389382 (Mest et al., 2001).

4.1. Effect of midaglizole

Midaglizole blocks both Kir6.2/SUR1 and Kir6.2ΔC currents with half-maximal inhibitory concentrations in the low micromolar range. This suggests that interaction of this drug with the sulphonylurea receptor subunit does not contribute to channel inhibition. Equivalent observations were previously made for the effect of the imidazoline phentolamine and the insulin-releasing imidazoline drug S-22068 on recombinant K_{ATP} channel (Proks and Ashcroft, 1997; Le Brigand et al., 1999). It seems most likely that midaglizole binds to a site located on the Kir6.2 subunit of the channel, although we cannot exclude the possibility that it interacts with a different protein, endogenously expressed in *Xenopus* oocytes, that regulates Kir6.2 activity. However, a recent study on the imidazoline cibenzoline demonstrated direct binding of the drug to the truncated Kir6.2 subunit (Mukai et al., 1998).

The IC_{50} value of 4.4 μM measured for inhibition of Kir6.2/SUR1 channels by midaglizole is in good agreement with the EC_{50} value of 24 μM determined for the stimulation of insulin secretion from MIN6 cells, a β-cell line (Mest et al., 2001; Table 1). This suggests that the insulin secretory capacity of the drug is a consequence of its ability to block K_{ATP} channels in this cell line. The EC_{50} for stimulation of insulin secretion from rat islets is slightly lower (0.2 μM) than the IC_{50} measured for inhibition of recombinant K_{ATP} channels (Mest et al., 2001; Table 1). It seems unlikely that this reflects a species

Table 1
EC_{50} values for stimulation of insulin release from rat islets and the MIN6 β-cell line and IC_{50} values for K_{ATP} current inhibition induced by midaglizole, LY397364 and LY389382

Drug	EC_{50} rat islets	EC_{50} MIN6	IC_{50} Kir6.2	IC_{50} Kir6.2/SUR1 (low affinity)
Midaglizole	200 nM[a]	24 μM[a]	3.8 μM	4.4 μM
LY397364	105 nM	300 nM	6.1 μM	880 nM
LY389382	310 nM[a]	1.1 μM[a]	700 nM	170 nM

[a] Data taken from Mest et al. (2001).

difference (rat islets vs. mouse Kir6.2) because mouse and rat Kir6.2 share almost complete sequence identity. Furthermore, since stimulation of insulin secretion from MIN6 cells has an EC_{50} that is 100 times higher than that from rat islets, it seems more likely that insulin secretion is somehow facilitated in intact islets.

The IC_{50} value obtained for midaglizole is close to that found for phentolamine ($IC_{50} = 1$ μM; Proks and Ashcroft, 1997). Thus, structures common to these two drugs may recognize the same Kir6.2 binding site.

4.2. Effects of LY397364 and LY389382

Both LY397364 and LY389382 blocked Kir6.2ΔC currents with affinities in the low micromolar range (6 and 0.7 μM, respectively), suggesting that these drugs interact directly with the Kir6.2 subunit. In both cases, the block was poorly reversible at high drug concentrations. These two drugs are derived from a common naphthol moiety. The main difference is in the position where the toluoyl or chlorobenzyl sidegroup is attached. On the other hand, these compounds vary considerably from the structure of the readily reversible midaglizole. It is therefore very difficult to determine which part of the molecule might be responsible for the poor reversibility of LY397364 and LY389382 block at high concentrations.

When Kir6.2 was coexpressed with SUR1, both a low-affinity site and high-affinity site were observed for inhibition by LY397364 and LY389382. Most of the block was mediated by interaction with the low-affinity site. For both drugs, this site was approximately five times more sensitive than that measured for Kir6.2ΔC currents. The simplest interpretation of this data is that the low-affinity site represents drug binding to Kir6.2, and that the presence of SUR1 either increases the affinity of the binding site, or enhances the efficacy with which drug binding is converted into channel closure. A similar effect is observed for ATP: channel inhibition by ATP is enhanced by the presence of SUR, although the ATP-binding site is thought to reside on Kir6.2 (Tucker et al., 1997; Tanabe et al., 2000). Additionally, inhibition of Kir6.2 currents by stilbene sulphonates shows similar enhancement in the presence of SUR (Proks et al., 2001). The fact that enhancement of inhibition was not observed for midaglizole suggests that LY397364 and LY389382 might interact with a different site on Kir6.2.

There appeared to be an additional high-affinity site for LY397364 and LY389382 present on SUR1. Inhibition associated with this site was generally irreversible, and its magnitude showed considerable variability between different oocytes. It was therefore not possible to obtain an accurate estimate of the affinity of this site, but it appears to be in the low nanomolar range. However, saturation of this site accounted for only about 30% of maximal channel inhibition and its pharmacological relevance is therefore not clear. The reason for the variability in the high-affinity block is unknown. One possibility is that the inhibition is indirect: for example, mediated by interaction of the drug with some cytosolic/membrane constituent that modulates channel rundown and whose concentration (or activity) varies between oocytes. Alternatively, modulators that influence channel activity (e.g. phospholipids) may also influence coupling between SUR and Kir6.2 and thereby the contribution of the LY397364 and LY389382 high-affinity site to channel inhibition.

4.3. Effect on insulin secretion

Half-maximal stimulation of insulin secretion from intact islets was produced by 0.1 μM LY397364, and from MIN6 cells by 0.3 μM LY397364. Thus, insulin secretion is more sensitive to the drug than the IC_{50} for inhibition of Kir6.2/SUR1 currents at the low-affinity site (0.88 μM). However, the high-affinity site had an affinity that lies in the low nanomolar range. Thus, we cannot exclude the possibility that block of K_{ATP} channel activity at this site may be responsible for the ability of LY397364 to stimulate insulin secretion. In the case of LY389382, stimulation of insulin secretion occurred with an EC_{50} of 0.3 μM LY389382 in intact islets, and an EC_{50} of 1.1 μM LY389382 in MIN6 cells (Mest et al., 2001). These values are similar to those observed for low-affinity inhibition of Kir6.2/SUR1 currents (0.17 μM), supporting the idea that stimulation of insulin secretion is primarily due to the block of K_{ATP} channel activity, and that this block is mediated by interaction of the drug with the Kir6.2 subunit. However, stimulation of insulin secretion by these compounds is glucose-dependent. The present study revealed no stimulation of insulin secretion from MIN6 cells in 3.3 mM glucose by up to 100 μM LY397364, a concentration that completely inhibits K_{ATP} channel activity. While this observation adds strong support for a K_{ATP} channel-independent action of this imidazoline on insulin secretion, it should be noted that a similar observation was made by Efanov et al. (2001b) when analyzing the effect of sulphonylureas on insulin secretion. These authors required considerably higher concentrations of sulphonylureas to stimulate insulin secretion in the presence of 3.3 mM glucose than in the presence of 16.7 mM glucose. An explanation could be that in the absence of glucose, most K_{ATP} channels are activated. Accordingly, a large proportion of the channels have to be inhibited by the drug before stimulation of insulin secretion occurs.

However, the related imidazoline BL11282 has been shown to stimulate insulin secretion from pancreatic islets, but not to modulate the electrical properties of rat pancreatic β-cells, suggesting that inhibition of K_{ATP} channel activity is not required for the insulinotropic action of this compound (Efanov et al., 2001b; see also Zaitsev et al., 1996). The former group also demonstrated stimulation of insulin

secretion by a further imidazoline, RX871024, from pancreatic β-cells of SUR1-deficient mice, adding more evidence for the K_{ATP} channel-independent effects of this class of compounds (Efanov et al., 2001a). Consequently, it has been suggested that these drugs can interact directly with the secretory machinery of the β-cell (Zaitsev et al., 1996).

In summary, our results demonstrate that all three compounds tested block the β-cell K_{ATP} channels with similar IC_{50} to those observed to stimulate insulin secretion in the presence of glucose (Table 1). While our results are consistent with the idea that these compounds stimulate insulin secretion via K_{ATP} channel inhibition, only future insulin secretion studies conducted on islets from K_{ATP} channel-deficient animals can produce conclusive evidence for K_{ATP} channel-independent modulation of insulin release by these three compounds. All three imidazolines exerted inhibitory effects on the Kir6.2 subunit which is common to K_{ATP} channels from various tissues, including heart muscle. Consequently, the potential for effects on extrapancreatic K_{ATP} channels makes these compounds less attractive for clinical use as insulin secretagogues, independent of the ultimate mechanism these drugs employ to stimulate insulin secretion.

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