Phentolamine block of K_{ATP} channels is mediated by Kir6.2

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ABSTRACT The ATP-sensitive K^+ -channel (K_{ATP} channel) plays a key role in insulin secretion from pancreatic β cells. It is closed both by glucose metabolism and the sulfonylurea drugs that are used in the treatment of noninsulin-dependent diabetes mellitus, thereby initiating a membrane depolarization that activates voltage-dependent Ca^{2+} entry and insulin release. The β cell K_{ATP} channel is a complex of two proteins: Kir6.2 and SUR1. The former is an ATP-sensitive K^+ -selective pore, whereas SUR1 is a channel regulator that endows Kir6.2 with sensitivity to sulfonylureas. A number of drugs containing an imidazoline moiety, such as phentolamine, also act as potent stimulators of insulin secretion, but their mechanism of action is unknown. We have used a truncated form of Kir6.2, which expresses independently of SUR1, to show that phentolamine does not inhibit K_{ATP} channels by interacting with SUR1. Instead, our results argue that phentolamine may interact directly with Kir6.2 to produce a voltage-independent reduction in channel activity. The single-channel conductance is unaffected. Although the ATP molecule also contains an imidazoline group, the site at which phentolamine blocks is not identical to the ATP-inhibitory site, because phentolamine block of an ATP-insensitive mutant (K185Q) is normal. K_{ATP} channels also are found in the heart where they are involved in the response to cardiac ischemia: they also are blocked by phentolamine. Our results suggest that this may be because Kir6.2, which is expressed in the heart, forms the pore of the cardiac K_{ATP} channel.

It has been known for many years that certain drugs that contain an imidazoline nucleus, including several classical α -adrenoreceptor antagonists, act as potent stimulators of insulin secretion (1–4). Good evidence exists that the insulinotropic effects of these drugs do not result from antagonism of α -adrenoreceptors, but rather from inhibition of ATP-sensitive K^+ -channels (K_{ATP} channels) in the β cell plasma membrane (2–6). The activity of K_{ATP} channels sets the β cell resting potential and their inhibition by imidazolines leads to membrane depolarization, activation of Ca^{2+} -dependent electrical activity, and a rise in $[Ca^{2+}]_i$ that triggers insulin release (7). One of the most potent of the imidazolines is phentolamine, which blocks native K_{ATP} currents in β cells half-maximally at 0.7 μ M when added to the intracellular solution (6).

In addition to their effects on insulin secretion, imidazolines have cardiovascular actions that are independent of α -adrenoreceptors. For example, phentolamine causes peripheral vasodilation, increases heart rate, and enhances myocardial contractility (8). It also increases the duration of the ventricular action potential, an effect that probably results from the ability of the drug to block cardiac K_{ATP} channels (9). The potency of inhibition ($K_i = 1 \mu$ M) is similar to that found for β cell K_{ATP} currents (9).

The mechanism by which imidazolines inhibit K_{ATP} currents is unknown. The pharmacology of imidazoline block of K_{ATP} channels does not match that of either of the major subtypes of imidazoline receptor (I_1 or I_2), which has led to the suggestion that the channel is associated with a novel receptor for imidazolines (10). It has been speculated that this receptor might form part of the K_{ATP} channel itself (6).

The K_{ATP} channel is a complex of two proteins: a pore-forming subunit, Kir6.2, and the sulfonylurea receptor, SUR1 (11, 12). The former acts as an ATP-sensitive K-channel pore whereas SUR1 is a channel regulator that endows Kir6.2 with sensitivity to drugs such as the inhibitory sulfonylureas and the K-channel opener diazoxide (13). We have explored whether phentolamine interacts with SUR1 or with Kir6.2, by studying the effect of phentolamine on the Kir subunit in the absence of the sulfonylurea receptor. Kir6.2 does not express functional K-ATP currents alone (11, 12). We therefore have examined the effect of phentolamine on a C-terminally truncated form of Kir6.2 in which the last 26 (Kir6.2 Δ C26) or 36 (Kir6.2 Δ C36) C-terminal amino acids have been deleted. This channel is able to express significant current in the absence of SUR1 (13).

METHODS

Molecular Biology. A 26 (or 36) amino acid C-terminal deletion of mouse Kir6.2 (GenBank D50581) was made by introduction of a stop codon at the appropriate residue using site-directed mutagenesis. Site-directed mutagenesis was carried out by subcloning the appropriate fragments into the pALTER vector (Promega). Kir6.2, rat Kir1.1a (GenBank X722341, ref. 14), and rat SUR1 (GenBank L40624, ref. 15) cRNAs were synthesized as previously described (16).

Electrophysiology. *Xenopus* oocytes were defolliculated and injected with ≈ 0.04 ng cRNA encoding wild-type (wt) Kir6.2 plus 2 ng SUR1 cRNA, or with ≈ 2 ng Kir6.2 Δ C26 cRNA, ≈ 2 ng Kir6.2 Δ C36 cRNA or ≈ 0.04 ng Kir1.1a cRNA. The final injection volume was ≈ 50 nl per oocyte. Isolated oocytes were maintained in modified Barth's solution (16) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 5 mM pyruvate. Currents were studied 1–4 days after injection. Macroscopic currents were recorded from giant inside-out patches (16–17) using an EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) at 20–24°C using 200–400 k Ω electrodes. The holding potential was 0 mV, and currents were evoked by repetitive 3-s voltage ramps from -110 mV to $+100$ mV. The mean current amplitude at -100 mV, measured in nucleotide-free solution immediately after patch excision, varied between 0.5 and 5 nA for wtKir6.2 coexpressed with SUR1, and was between 0.2 and 1 nA for Kir6.2 Δ C26 currents. Current and voltage signals were filtered at 5 kHz and stored on digital audio tape. Currents subsequently were

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: K_{ATP} channel, ATP-sensitive potassium channel; wt, wild type.

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FIG. 1. Effects of imidazolines on wtK-ATP currents and Kir6.2ΔC26 currents. (A and B) Macroscopic currents (Upper) and associated current-voltage relations (Lower) recorded from two different inside-out patches in response to a series of voltage ramps from -110 to $+100$ mV. Oocytes were coinjected with cRNAs encoding wtKir6.2 and SUR1 (A), or Kir6.2ΔC26 (B). The holding potential was 0 mV. (Upper) The dotted line indicates the zero current level. Phentolamine ($100 \mu\text{M}$) was added to the intracellular (bath) solution as indicated by the solid line. (Lower) 1, control; 2, $100 \mu\text{M}$ phentolamine.

digitized at 5 kHz (filter, 2 kHz) using a Digidata 1200 Interface and pClamp 6.0 software (Axon Instruments, Foster City, CA), for analysis. Single-channel currents were recorded from inside-out patches at -60 mV and digitized at 10 kHz (filter, 5 kHz).

The pipette (external) solution contained 140 mM KCl, 1.2 mM MgCl_2 , 2.6 mM CaCl_2 , and 5 mM Hepes (pH 7.4). The intracellular (bath) solution contained 107 mM KCl, 10 mM EGTA, 2 mM MgCl_2 , 1 mM CaCl_2 , and 10 mM Hepes (pH 7.2 with KOH; final $\text{K}^+ \approx 140$ mM). Exchange of solutions was achieved by positioning the patch electrode in the mouth of one of a series of adjacent inflow pipes containing the test solutions.

HEK293 cells were cultured and transiently transfected with the pcDNA3 vector (Invitrogen) containing the coding sequence of Kir6.2ΔC36 using Lipofectamine (GIBCO/BRL) (12, 13). We used Kir6.2ΔC36, rather than Kir6.2ΔC26, as it expresses larger currents. Whole-cell currents were studied 48 – 72 hr after transfection. The pipette solution contained 107 mM KCl, 1.2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM EGTA, 5 mM Hepes (pH 7.2 with KOH; total $\text{K} \approx 140$ mM) and 0.3 mM ATP. The bath solution contained 5.6 mM KCl, 140 mM NaCl, 2.6 mM CaCl_2 , 1.2 mM MgCl_2 , and 5 mM Hepes (pH 7.4). Currents were evoked by ± 20 mV 200 -ms depolarizations from a holding potential of -70 mV.

Data Analysis. All data is given as mean $\pm$ one SEM. The symbols in the figures indicate the mean, and the vertical bars indicate 1 SEM. The slope conductance (G) was measured by fitting a straight line to the data between -20 mV and -100 mV; the average of 3 – 5 consecutive ramps was calculated in each solution. K_{ATP} currents commonly decline in amplitude after patch excision, at a variable rate (7, 13). To control for this rundown, test solutions were alternated with control solutions, and G was expressed as a fraction of the slope conductance in

control solution (G_c). For Kir6.2/SUR1 currents, phentolamine dose-response relationships were fit to the Hill equation: $100 G/G_c = B + (100 - B)/(1 + ([\text{phentolamine}]/K_i)^h)$, where $[\text{phentolamine}]$ is the phentolamine concentration, K_i is the drug concentration at which inhibition is half maximal, h is the slope factor (Hill coefficient), and B is the background current that is not blocked by $100 \mu\text{M}$ phentolamine. Kir6.2ΔC26 currents were smaller than Kir6.2/SUR2 currents (13). Consequently, the background current contributes a greater fraction of the total current and, because of rundown, this fraction increases throughout the course of the experiment (i.e., B is not constant). Therefore the background current was subtracted from Kir6.2ΔC26 currents and B was set to zero. The background current for Kir6.2ΔC26 currents amounted to 145 ± 35 pS ($n = 6$) and for wild-type currents was 175 ± 45 pS ($n = 6$).

Statistical significance was tested using Student's t test. Single-channel currents were analyzed using a combination of pClamp and in-house software. Single-channel current amplitudes were calculated from an all-points amplitude histogram. For measurements of open and closed times, events were detected using a 50% threshold level method, and a burst was defined as two openings separated by an interval of <0.6 ms (twice the mean short closed time).

RESULTS

We first recorded wt K_{ATP} currents from giant inside-out patches excised from *Xenopus* oocytes coinjected with wt-Kir6.2 and SUR1. As previously described (15), the patch conductance was low in the cell-attached configuration but increased spontaneously upon excision of the patch into a nucleotide-free solution. These currents were blocked by 1 mM ATP (15), demonstrating that oocytes coinjected with wt-

Kir6.2 and SUR1 express ATP-sensitive K-currents. Similar results were obtained for Kir6.2 Δ C26 and Kir6.2 Δ C36 currents, confirming that Kir6.2 has an intrinsic ATP-inhibitory site (13).

Application of 100 μ M phentolamine to the intracellular solution reversibly inhibited wtK_{ATP} currents by $97.1 \pm 0.7\%$ ($n = 7$) (Fig. 1A). A similar degree of inhibition ($91 \pm 3.9\%$, $n = 6$) was observed for Kir6.2 Δ C26 currents (Fig. 1B) and for Kir6.2 Δ C36 currents (data not shown). In both cases the block by phentolamine was voltage-independent.

The relationship between phentolamine concentration and the amplitude of the wtK_{ATP} current is given in Fig. 2A. It is clear that the dose-response curve can be well fit by a single-site model, with a mean K_i of $1.13 \pm 0.38 \mu$ M and a Hill coefficient of 1.28 ± 0.11 ($n = 6$). A similar relationship was found for Kir6.2 Δ C26 currents (Fig. 2B), and the mean K_i ($0.77 \pm 0.16 \mu$ M, $n = 6$) and Hill coefficient (1.14 ± 0.38) were not significantly different from those of wtK_{ATP} currents. These data suggest that the Kir6.2 subunit may possess an intrinsic phentolamine inhibitory site. The fact that the Hill coefficient was close to unity also suggests that only a single phentolamine molecule needs to interact with the channel to cause inhibition. It is believed that four Kir6.2 subunits come together to form the pore (18, 19), but we do not know whether each Kir6.2 subunit possesses its own phentolamine binding site, or whether there is a single site with residues contributed by all four subunits. The data also indicate that coexpression with SUR1 does not enhance the phentolamine sensitivity of Kir6.2, in contrast to what is observed for the inhibitory effects of ATP (13).

To exclude the possibility that truncated Kir6.2 couples to an endogenous protein in the *Xenopus* oocyte that endows phentolamine sensitivity, we also examined the effects of the drug on Kir6.2 Δ C36 currents expressed in the mammalian cell line HEK293. Whole-cell currents recorded from HEK293 cells transfected with Kir6.2 Δ C36 increased with time after establishment of the whole-cell configuration when dialyzed intracellularly with 0.3 mM ATP. This increase in current reflects the washout of endogenous ATP (7, 13). Washout-currents were blocked by $92.2 \pm 1.8\%$ by 100 μ M extracellular phentolamine (Fig. 3A), a value similar to that found when Kir6.2 Δ C26 was expressed in oocytes. The slower time course of the block when phentolamine is applied to the extracellular rather than intracellular membrane surface is observed for native β cells also (unpublished observations). This presumably reflects the access of phentolamine to its binding site and suggests it may lie on the inner side of the membrane.

We next examined the effect of phentolamine on wtK_{ATP} currents and Kir6.2 Δ C26 currents at the single-channel level (Fig. 4). As previously reported (13), no difference in the unitary conductance of wtK_{ATP} and Kir6.2 Δ C26 channels was found (Table 1). However, the kinetics of Kir6.2 Δ C26 currents were faster (Table 1). For wtK_{ATP} currents, the open time distribution was fit by a single exponential, with a mean time constant of 1.49 ms. The closed time distribution was best fit by the sum of two exponentials, with mean time constants of 0.32 ms and 6.2 ms, respectively. These values are similar to those previously reported for native and wtK_{ATP} channels (7, 12). As seen in Fig. 4A, K_{ATP} channel openings occurred in bursts, which were separated by longer closed intervals. The short closed time is primarily determined by openings within a burst and the long closed time reflects the inter-burst intervals. The mean short closed time of Kir6.2 Δ C26 currents was not significantly different from that of wtK_{ATP} channels; however, the mean burst duration and the mean open time were significantly shorter (Table 1). This produces a decrease in the channel open probability, which may, in part, explain why we observed smaller amplitude macroscopic currents when Kir6.2 Δ C26 was expressed alone than when it was coexpressed with SUR1 (13).

Phentolamine did not alter the amplitude of either wtK_{ATP} or Kir6.2 Δ C26 single-channel currents, indicating that it does not act as an open channel blocker (Table 1; Fig. 4). However, it markedly decreased the channel activity. For both wtK_{ATP} currents and Kir6.2 Δ C26 currents this effect resulted primarily from a reduction in the mean long closed time.

We next examined whether the site at which phentolamine mediates K_{ATP} channel inhibition was identical with that involved in ATP block, by examining the effect of phentolamine on a mutant form of Kir6.2 (Kir6.2 Δ C26-K185Q), which has much lower ATP sensitivity. In this mutant, the K_i for ATP block is reduced from 106 μ M to 4.2 mM (13). However, 100 μ M phentolamine blocked Kir6.2 Δ C26-K185Q currents as effectively as Kir6.2 Δ C26 currents (Fig. 3C). This argues that the phentolamine-inhibitory site is not identical with that for ATP, although it remains possible that both sites may share certain residues.

Finally, we explored whether phentolamine is able to inhibit Kir1.1a, a related Kir channel that shares $\approx 45\%$ sequence identity with Kir6.2. As shown in Fig. 3B, 100 μ M phentol-

FIG. 2. Concentration-dependence of phentolamine block of wtK_{ATP} currents and Kir6.2 Δ C26 currents. Mean dose-response relationships for phentolamine block of wtKir6.2/SUR1 currents (A, $n = 6$) or Kir6.2 Δ C26 currents (B, $n = 6$). Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a percentage of the mean (G_c) of that obtained in control solution before and after exposure to the drug. The lines are the best fit of the mean data to the Hill equation (see *Methods*). For wtKir6.2/SUR1 currents, $K_i = 1.15 \mu$ M, $h = 1.33$, and $B = 2.93$. For Kir6.2 Δ C26 currents, $K_i = 0.72 \mu$ M, $h = 1.1$; the current in the presence of 100 μ M phentolamine was taken as the background current and was subtracted (this corresponded to $<10\%$ of the total current).

Ai. Kir 6.2 Δ C36

B. Kir 1.1

Aii.

C.

FIG. 3. (A) Phentolamine block of K-ATP currents expressed in HEK293 cells. (Ai) Whole-cell currents recorded from an HEK293 cell transfected with cDNA encoding Kir6.2 Δ C36 in response to alternate ± 20 mV pulses from a holding potential of -70 mV. (Aii) Mean whole-cell current amplitudes recorded from HEK293 cells transfected with cDNA encoding Kir6.2 Δ C36 or pcDNA3 alone, in control solution and after the addition of $100 \mu\text{M}$ phentolamine. Cells were dialyzed with 0.3 mM ATP. (B and C) Selectivity of phentolamine block. (B) Macroscopic currents recorded in response to a series of voltage ramps from -110 to $+100$ mV from an inside-out patch excised from an oocyte injected with Kir1.1a cRNA. The holding potential was 0 mV . The dotted line indicates the zero current level. Phentolamine ($100 \mu\text{M}$) was added to the intracellular (bath) solution as indicated by the solid line. (C) Percentage inhibition of macroscopic currents by intracellular ATP (empty bars) or phentolamine (filled bars). Currents are expressed as a fraction of their amplitude before addition of the inhibitor. Inside-out patches were excised from oocytes injected with cRNA encoding wtKir6.2 Δ C26 ($n = 6$) or wtKir6.2 Δ C26-K185Q ($n = 6$).

amine was without significant effect on Kir1.1a. The mean conductance in the presence of the drug was $99.0 \pm 2.7\%$ ($n = 6$) of that in control solution.

DISCUSSION

Our results demonstrate that the imidazoline phentolamine does not inhibit the β cell wtK_{ATP} channel by binding to the sulfonylurea receptor SUR1. This explains why imidazolines do not displace [^3H]glibenclamide binding to β cell membranes (20). It also is consistent with the finding that the sensitivity of the native β cell K_{ATP} channel to imidazolines, unlike that to sulfonylureas (21), does not decline after patch excision or after mild proteolysis of the intracellular membrane (5).

The fact that phentolamine blocks Kir6.2 Δ C26 currents as potently as Kir6.2/SUR1 currents suggests that the binding site for phentolamine resides on Kir6.2, or on a separate protein,

endogenously expressed in *Xenopus* oocytes, which regulates Kir6.2 activity. Although we cannot completely exclude the latter possibility, we favor the idea that Kir6.2 is itself sensitive to phentolamine. This is because the extent of block of Kir6.2 Δ C26 currents did not depend on the current size, as might be expected if an endogenous oocyte protein were to mediate the inhibitory effect of the drug. The observation that Kir6.2 Δ C36 currents also were blocked by phentolamine in HEK293 cells also makes it less likely that phentolamine sensitivity is conferred by an endogenous subunit as this protein also would have to be expressed in HEK293 cells. Because both the native β cell (6) and cardiac K_{ATP} channel (9) are blocked by phentolamine with a potency similar to the one we report for Kir6.2 Δ C26 currents, any imidazoline receptor distinct from Kir6.2 itself also would have to be expressed in these cells. Finally, a brief report that Kir6.2 can be radiolabeled by cibenzoline, another imidazoline that blocks K_{ATP}

Table 1. Properties of wtK_{ATP} and Kir6.2 Δ C26 single-channel currents

	Mean open time, ms	Mean short closed time, ms	Mean long closed time, ms	Mean burst duration, ms	Mean openings per burst	i (pA, at -60 mV)
wtK _{ATP} currents						
Control ($n = 3$)	1.4 ± 0.3	0.32 ± 0.02	6.2 ± 0.7	4.3 ± 0.8	3.3 ± 0.3	4.4 ± 0.2
Phentolamine ($n = 3$)	1.3 ± 0.2	0.34 ± 0.02	17.5 ± 6.1	4.7 ± 1.0	3.3 ± 0.3	4.3 ± 0.2
Kir6.2 Δ C26 currents						
Control ($n = 3$)	0.8 ± 0.1	0.29 ± 0.03	3.8 ± 1.5	2.1 ± 0.4	2.5 ± 0.2	4.3 ± 0.2
Phentolamine ($n = 3$)	0.7 ± 0.1	0.31 ± 0.01	11.4 ± 3.3	2.0 ± 0.4	2.5 ± 0.1	4.3 ± 0.3

i, single-channel current amplitude (at -60 mV).

FIG. 4. Effects of phentolamine on single-channel currents. Single-channel currents recorded at -60 mV in the presence or absence of $1 \mu\text{M}$ intracellular phentolamine from an inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1 cRNAs (A) or with Kir6.2ΔC26 cRNA (B). The arrow indicates the zero current level.

channels and stimulates insulin secretion (22), has appeared (23). These data argue that phentolamine interacts directly with Kir6.2 to close the pore.

Although both ATP and phentolamine block Kir6.2, the site at which they do so does not appear to be composed of identical amino acids, because mutation of K185Q markedly decreases the ATP sensitivity (13) yet was without significant effect on phentolamine block. It remains possible, however, that the binding sites for ATP and phentolamine may share some of the same residues. The fact that the adenosine molecule includes an imidazoline moiety favors this possibility.

Kir6.2 is expressed in β cells, heart, brain, and skeletal muscle and therefore may serve as the pore-forming subunit of the K_{ATP} channel in all of these tissues (12). By contrast, the sulfonylurea receptor, which acts as the regulatory subunit of the K_{ATP} channel, is different in cardiac muscle, being SUR2A rather than SUR1 (24). Our demonstration that Kir6.2 serves as the target for imidazolines therefore explains why native cardiac and β cell K_{ATP} channels share a similar sensitivity to phentolamine. Imidazolines are being explored as novel oral hypoglycemic agents for the treatment of noninsulin-dependent diabetes. Our results indicate that this strategy requires careful consideration because these drugs are likely to have side effects in other tissues where Kir6.2 is expressed, such as heart, brain, and skeletal muscle. Finally, we point out that in addition to the sulfonylureas and the imidazolines, a range of other drugs inhibit K_{ATP} channels: these include

barbiturates, antimalarial agents such as quinine, and certain plant extracts (25). It will be important to consider whether these drugs mediate their effects by interaction with Kir6.2 rather than SUR1, as this has obvious implications for their tissue specificity.

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