Interaction of the Cytosolic Domains of the Kir6.2 Subunit of the K_{ATP} Channel Is Modulated by Sulfonylureas

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The NH_2- and COOH-termini of the ATP-sensitive potassium (K_{ATP}) channel pore-forming subunit, Kir6.2, both lie intracellularly and interact with one another. To study this interaction, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were fused to the NH_2- and COOH-termini of Kir6.2, respectively (CFP-Kir6.2-YFP). These fluorescent proteins have sufficient spectral overlap to allow distance-dependent fluorescence resonance energy transfer (FRET). When CFP-Kir6.2-YFP was expressed in human embryonic kidney cells and illuminated at 440 nm to excite CFP, significant fluorescence was recorded at 535 nm, the peak of the YFP emission spectrum. This indicated that FRET was occurring and thus that the NH_2- and COOH-termini of Kir6.2 lie in close proximity to one another. The emission ratio, F_{535}/F_{480}, was increased by co-expression of SUR2A, but not SUR1, suggesting that SUR2A but not SUR1 influences the Kir6.2 NH_2- and COOH-terminal interaction. This interaction was reduced by the sulfonylureas tolbutamide and gliclazide, but not by the pore blocker barium. The properties of the tolbutamide response indicate that the drug disrupts the interaction between the NH_2- and COOH-termini of Kir6.2 by binding to a low-affinity site on Kir6.2.
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ATP-sensitive potassium (K_{ATP}) channels couple cellular metabolism to electrical activity and thereby play important roles in many cell types. In pancreatic β-cells, closure of these channels in response to glucose metabolism or sulfonylureas produces a depolarization that ultimately leads to insulin secretion. K_{ATP} channels are heteromeric (4:4) complexes of two types of protein subunit: an inwardly rectifying potassium channel subunit, which belongs to the Kir6.x subfamily, and a sulfonylurea receptor (SUR; a member of the ATP-binding cassette family). The resulting potassium channel is sensitive to intracellular nucleotides, phospholipids, and several kinds of therapeutically important drugs, including sulfonylureas and potassium channel openers. Different types of SUR subunits endow the channel with differential sensitivity to sulfonylureas: SUR1 possesses a high-affinity binding site for tolbutamide, gliclazide, and glibenclamide (1,2), whereas SUR2A binds glibenclamide, but not tolbutamide or gliclazide, with high affinity. A low-affinity site for sulfonylureas is also present on Kir6.2 (1). The K_{ATP} channel of the pancreatic β-cell plasma membrane is composed of Kir6.2 and SUR1 subunits.

The Kir6.2 subunit has two transmembrane domains with NH_2- and COOH-termini that lie intracellularly. Previous studies have provided biochemical evidence for regions that mediate physical interaction between these two intracellular domains (3,4). Furthermore, the interaction domains include residues responsible for functional channel assembly (3), and for regulation by ATP (5), protons (6), and phospholipids (7).

In this study, we describe the use of fluorescence resonance energy transfer (FRET) to explore the interaction between the NH_2- and COOH-termini of Kir6.2. FRET occurs through dipole-to-dipole interactions between fluorophores when the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The process is dependent on the distance between the fluorophores and, in the case of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (8,9), occurs when the intermolecular distance is <10 nm. A decrease in this distance results in an increase in FRET, which is detected as a decrease in the donor (CFP) fluorescence and a concomitant increase in the acceptor (YFP) fluorescence. Changes in FRET can be used to measure interactions between proteins, or between domains of a single protein, that have been tagged with CFP and YFP. The results of our study reveal that the distal NH_2- and COOH- termini of Kir6.2 lie in close proximity, and that drugs that mediate channel inhibition via Kir6.2 may do so by modulating the interaction between these two domains.

RESEARCH DESIGN AND METHODS

Molecular biology and mammalian expression. The vectors pECFP-C1 and pEYFP-N1 (Clontech) were used to construct fluorescent Kir6.2 fusion proteins. For labeling both termini of Kir6.2, a hybrid vector (pECFP-EYFP) was constructed by replacing the smaller *Bgl*II/*Afl*II fragment from pECFP-N1 with one from pEYFP-N1 that contains a component of the multiple cloning site, the EYFP sequence, and the polyadenylation signal. Mouse Kir6.2 cDNA

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CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; HEK cell, human embryonic kidney cell; K_{ATP} channel, ATP-sensitive potassium channel; PMT, photomultiplier; SUR, sulfonylurea receptor; YFP, yellow fluorescent protein.

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(Genbank D50581) was ligated to the *Bsp*E1/*Bam*HI sites of pECFP-N1 and pECFP-EYFP.

Human embryonic kidney (HEK) cells were cultured in minimum essential medium containing glutamax (Gibco), supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% sodium pyruvate, and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transiently transfected with CFP or YFP alone, or with CFP-Kir6.2 or CFP-Kir6.2-YFP either by themselves or together with rat SUR1 (GenBank L40624) or SUR2A (GenBank D83598) cDNA using Fugene 6 reagent (Roche). They were seeded onto borosilicate glass cover slips and studied 1–6 days later.

Fluorescence and electrophysiological measurements. Single cells were visualized using a Nikon Diaphot microscope with a Fluor ×40 oil-immersion objective lens. Cells were excited using a Xenon arc lamp (Nikon) at either 440 nm (CFP) or 470 nm (YFP) (Omega optical filters). Emission at 480 nm and 535 nm was detected with a pair of photomultipliers (PMTs) (Hamamatsu R6095). Signals were amplified, filtered at 10 Hz (Cairn), and digitized at 1 kHz using a Digidata 1200 analog-to-digital converter (Axon). A Zeiss Axioplan2/LSM 510 confocal microscope was also used in some experiments for fluorescence imaging and acceptor bleaching.

The whole-cell configuration of the patch clamp technique was used to record K_{ATP} currents and clamp the intracellular pH, Ca^{2+}, and ATP concentrations. Patch pipettes were fabricated from thin-walled borosilicate glass capillaries (Harvard), coated with Sylgard (Dow Corning) close to their tips and heat polished. Currents were recorded with an EPC-7 patch clamp amplifier, filtered at 3 kHz and digitized at 1 kHz (Digidata 1200, Axon). The holding potential was –70 mV, and 100-ms pulses to –50 mV were applied at 1 Hz. The pClamp 8 software package (Axon) was used to generate voltage-clamp protocols and to acquire and analyze the digitized data.

Solutions and drugs. The bath solution contained (in mmol/l) 140 NaCl, 5.6 KOH, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4 with HCl), and $BaCl_2$, as indicated. The patch pipette solution contained (in mmol/l) 100 KCl, 40 KOH, 10 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 0.3 K_2ATP, and 10 HEPES (pH 7.2 with HCl). Stock solutions of tolbutamide (0.1 mol/l in 0.15 mol/l NaOH) and gliclazide (0.1 mol/l in 0.1 mol/l NaOH) were prepared and diluted as required, and the pH was readjusted if necessary. Cells were continuously perfused using a gravity-feed system (0.5 ml/min), and experiments were performed at room temperature (22–25°C).

Data analysis. Fluorescence intensity was measured as the arbitrary voltage output from the PMT amplifiers. The emission spectrum of CFP and YFP show some overlap, so that a component of the signal detected at 535 nm is CFP emission rather than the entire signal representing YFP emission alone. The extent of this CFP "bleedthrough" was quantified by measuring the relative amount of 535 to 480 nm emission when CFP was expressed in HEK cells by itself. This value (0.64) was then used to correct the 535-nm signal measurement in experiments carried out with CFP-Kir6.2 and CFP-Kir6.2-YFP, using the following equation: corrected $F_{535} = F_{535} - (0.64 \times F_{480})$. The values for F_{535} reported here represent the corrected values.

For analysis and presentation in the figures, fluorescence and electrophysiological data were resampled at 1 Hz. Data are expressed as means ± 1 SE.

RESULTS AND DISCUSSION

Recombinant K_{ATP} channels consisting of CFP-Kir6.2 or CFP-Kir6.2-YFP (Fig. 1*A*) with or without SUR1 or SUR2A were expressed in HEK cells and illuminated at 440 nm, the excitation wavelength of the donor fluorophore CFP. Fluorescence was recorded at both 535 nm, the peak emission wavelength of the acceptor fluorophore YFP, and at 480 nm, the peak emission wavelength of CFP. The extent of FRET was quantified as the fluorescence ratio F_{535}/F_{480}.

Figure 1*B* shows the F_{535}/F_{480} ratio recorded for each combination of subunits studied. This value was negligible for CFP-Kir6.2 (which lacks the YFP fluorophore), demonstrating that our method of correction for CFP "bleedthrough" was effective. In contrast, cells expressing CFP-Kir6.2-YFP showed significant FRET (Fig. 2*B*). This result indicates that CFP and YFP lie within a few nanometers of each other. Because CFP is attached to the NH_2-terminus of Kir6.2 and YFP to the COOH-terminus, the distal parts of the intracellular domains of Kir6.2 must also lie in close

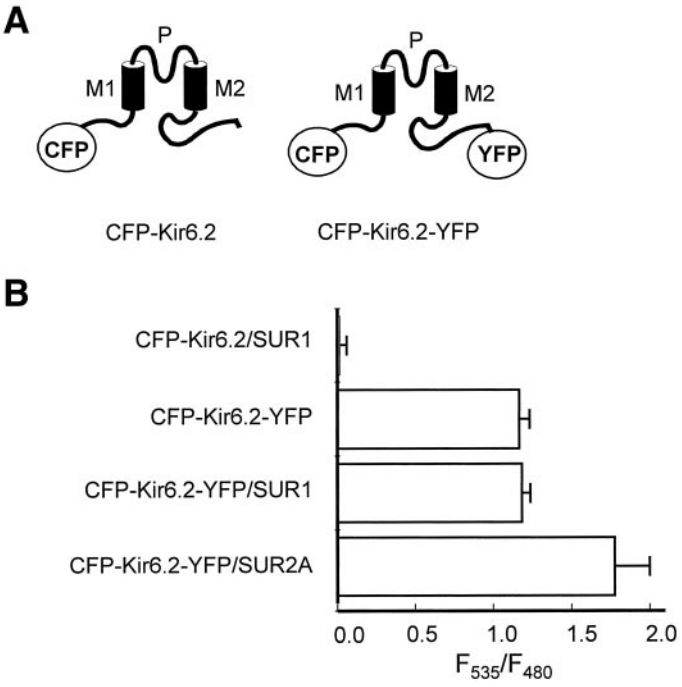

FIG. 1. *A*: CFP and YFP were fused to the NH_2- and COOH-termini of Kir6.2, respectively. *B*: Ratio of CFP emission (480 nm) to YFP emission (535 nm) obtained for the indicated constructs, after correction for CFP emission at 535 nm (see RESEARCH DESIGN AND METHODS). Constructs were expressed in HEK cells. M1 and M2, first and second transmembrane domains; P, pore loop.

proximity. Co-expression with SUR1 did not affect the extent of FRET between CFP and YFP, whereas co-assembly with SUR2A increased the amount of FRET significantly. This suggests that SUR2A, but not SUR1, influences the interaction between the NH_2- and COOH-termini of Kir6.2 detected by the FRET pair. The increase in FRET produced by SUR2A may result either from a reduction in the distance between the NH_2- and COOH-termini of Kir6.2 or from a change in the relative orientation of the fluorophores.

To confirm that the F_{535}/F_{480} ratio represents FRET, we examined the effect of selectively bleaching the acceptor fluorophore (YFP) on the fluorescence intensity of the donor fluorophore (CFP). This is predicted to produce an increase in CFP emission, as the signal is no longer quenched by fluorescence resonance energy transfer to YFP. We observed a significant increase in CFP emission that accompanied the YFP decrease when a small region of the cell membrane was illuminated at 514 nm at high intensity using a confocal microscope (data not shown). This observation provides good evidence that FRET occurs between CFP and YFP when they are fused to the NH_2- and COOH- termini of Kir6.2.

We next examined the effect of tolbutamide on the interaction between the intracellular domains of Kir6.2, using concentrations between 10 μmol/l and 10 mmol/l, which spans the range of high- and low-affinity binding sites (1). To determine if tolbutamide had any nonspecific effects on CFP or YFP, we first expressed the fluorescent proteins by themselves in HEK cells, and recorded fluorescence intensities at different drug concentrations. At a concentration of 3 mmol/l, tolbutamide quenched both CFP (480 nm) and YFP (535 nm) fluorescence in a con-

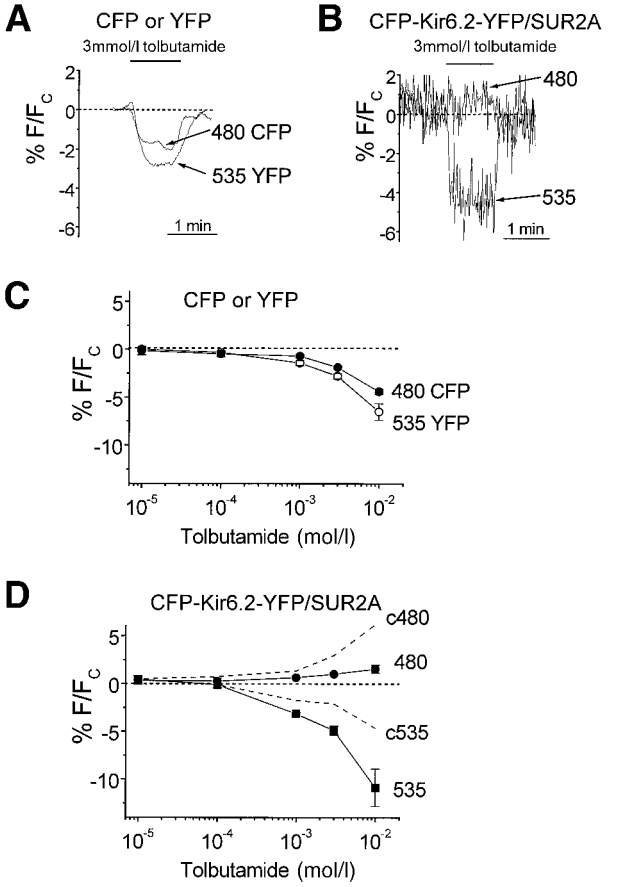

FIG. 2. ***A*: Representative recordings of fluorescence changes at 480 nm from a HEK cell expressing CFP, and of fluorescence changes at 535 nm from a different cell expressing YFP, in response to external tolbutamide (3 mmol/l). *B*: Representative recordings of the simultaneous fluorescence changes at 480 and 535 nm from a HEK cell expressing CFP-Kir6.2-YFP and SUR2A. Tolbutamide (3 mmol/l) was applied to the bath solution as indicated by the bar. *C*: Relation between tolbutamide concentration and the magnitude of the fluorescence change at F_{480} from cells expressing CFP (●; $n = 5$) or at F_{535} from cells expressing YFP (○; $n = 5$) alone. *D*: Relation between tolbutamide concentration and the magnitude of the fluorescence change at F_{480} (●) and F_{535} (■) recorded simultaneously from cells expressing CFP-Kir6.2-YFP and SUR2A ($n = 5$). The dashed lines labeled c480 and c535 indicate the data after correction for quenching by tolbutamide (see text for details). The fluorescence in the presence of tolbutamide (F) is given as a percentage of that in control solution (F_c) and expressed as the percentage change [i.e., ($F/F_c \times 100$) − 100].**

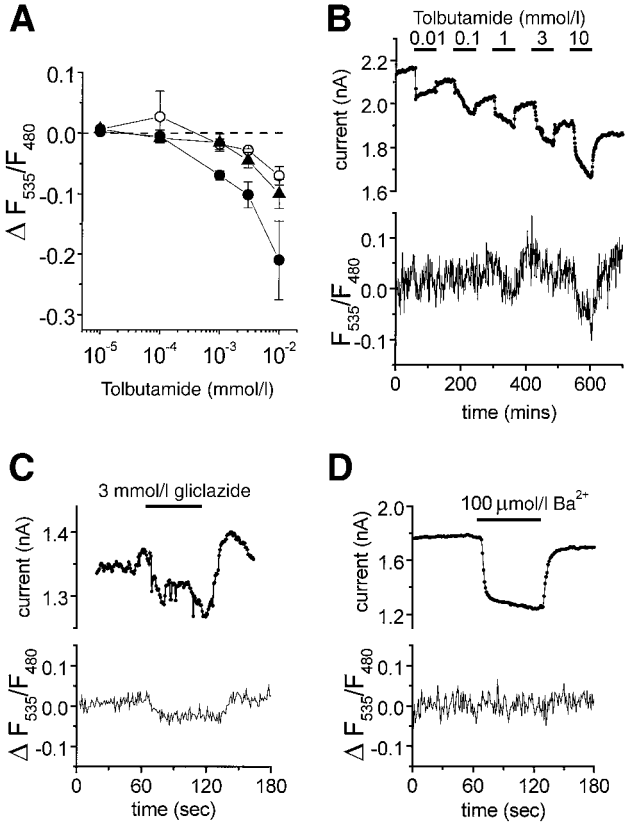

FIG. 3. ***A*: Relation between tolbutamide concentration and the magnitude of the change in the fluorescence ratio F_{535}/F_{480}, for cells expressing CFP-Kir6.2-YFP alone (○, $n = 5$) or in combination with SUR1 (▲, $n = 5$) or SUR2A (●, $n = 5$). *B*: Whole-cell currents recorded in response to 20-mV depolarizations from −70 mV from a cell expressing CFP-Kir6.2-YFP and SUR1. Tolbutamide was applied during the bars at the concentrations indicated. *C* and *D*: Representative recordings of whole-cell currents in response to 20-mV depolarizations from −70 mV (above) and changes in the fluorescence ratio F_{535}/F_{480} (below) from two different cells expressing CFP-Kir6.2-YFP and SUR1. Gliclazide (3 mmol/l; *C*) or barium (100 μmol/l; *D*) was applied during the periods indicated by the bars.**

centration-dependent manner (Fig. 2*A* and *C*). This was also the case for CFP-Kir6.2 (data not shown). In contrast, when 3 mmol/l tolbutamide was applied to cells coexpressing CFP-Kir6.2-YFP and SUR2A, the 535-nm signal was reduced, whereas the 480-nm emission was unchanged (Fig. 2*B*). Mean concentration-response curves are given in Fig. 2*D* (solid lines), and reveal that there is a small concentration-dependent increase in emission at 480 nm (CFP donor) and a decrease in emission at 535 nm (YFP acceptor). The extent of the decrease in the 535 nm emission was greater than that of quenching alone (compare Fig. 2*C* and *D*). We corrected for quenching by dividing the mean data points by the fraction of fluorescence intensity remaining at the equivalent tolbutamide concentration for CFP or YFP expressed alone (Fig. 2*C*). The corrected data (Fig. 2*D*, dashed lines) reveal that tolbutamide produces a concentration-dependent increase in CFP fluorescence and an equivalent decrease in YFP fluorescence. This indicated that 3 mmol/l tolbutamide reduces the extent of FRET between CFP and YFP, and thus that it induces a conformational change in Kir6.2.

The sensitivity of wild-type K_{ATP} channels to tolbutamide varies with the type of sulfonylurea receptor (1). We therefore compared the effect of the drug on K_{ATP} channels consisting of CFP-Kir6.2-YFP expressed alone or combination with SUR1 or SUR2A. The concentration dependence of the fluorescence change was similar for all channels (Fig. 3*A*). The larger magnitude of the changes observed for CFP-Kir6.2-YFP/SUR2A may be attributable to the different level of FRET in control solution (Fig. 1*B*), because the relation between FRET efficiency and CFP-YFP distance is nonlinear. The concentrations of tolbutamide required to produce a change in FRET are similar to those that produce channel inhibition by interaction with the low-affinity site on Kir6.2 (1). This suggests that tolbutamide binding to SUR does not alter the interaction between the NH_2- and COOH-termini of Kir6.2 detected by the FRET pair, and that the effects of tolbutamide we observed were mediated via the Kir6.2 subunit.

Low concentrations of tolbutamide (10 and 100 μmol/l) decreased CFP-Kir6.2-YFP/SUR1 currents significantly, but did not produce a change in FRET (Fig. 3*B*). This

suggests that the changes in FRET we observed were not the result of channel closure, but instead reflect a conformational change produced by tolbutamide binding to the low-affinity site on Kir6.2. In support of this idea, external barium (100 μmol/l), which decreases K_{ATP} current by plugging the pore of the channel (by acting as an open channel blocker), did not affect FRET (Fig. 3*D*). This result also indicates that the fluorescence changes we detected were not the result of changes in potassium flux through the Kir6.2 pore, which conceivably might have altered the local ionic environment of the fluorescent protein.

The sulfonylurea gliclazide resembles tolbutamide in that it inhibits Kir6.2/SUR1 channels by interaction with both high- and low-affinity sites located on SUR1 and Kir6.2, respectively (2). Figure 3*C* shows that gliclazide (3 mmol/l) also produced a decrease in FRET. This indicates that the effect of tolbutamide on the interaction between the NH_2- and COOH-termini of Kir6.2 can be mimicked by other sulfonylureas.

In the presence of SUR1, Kir6.2 is trafficked to the surface membrane (10). We confirmed that this was also the case for CFP-Kir6.2-YFP/SUR1 by confocal microscopy (data not shown). Because little fluorescence was found within the cell, most of the signal must come from the surface membrane. However, we also observed that when CFP-Kir6.2-YFP was expressed alone, it was principally located in internal membranes. This finding is in agreement with the lack of cell-attached channel activity or whole-cell currents detected from cells expressing this construct. Because substantial FRET was observed for CFP-Kir6.2-YFP in the absence of SUR1 (Fig. 1*B*), the NH_2- and COOH-termini of Kir6.2 must be capable of interacting with one another in intracellular membranes. The fact that this interaction can be modulated by extracellularly applied tolbutamide (Fig. 3*A*) is consistent with the lipophilicity of the drug.

CONCLUSION

In summary, the data presented here provide evidence that the distal parts of the NH_2- and COOH-termini of Kir6.2 lie in close proximity to one another. Co-assembly with SUR2A, but not SUR1, increases the extent of FRET, suggesting that SUR2A, but not SUR1, causes a conformational change in Kir6.2 that enhances this interaction. In contrast, the sulfonylurea tolbutamide disrupts the interaction between the NH_2- and COOH-termini by binding to a low-affinity site on Kir6.2.

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