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Intrinsic Sensitivity of Kir1.1 (ROMK) to Glibenclamide in the Absence of SUR2B

IMPLICATIONS FOR THE IDENTITY OF THE RENAL ATP-REGULATED SECRETORY K^+ CHANNEL*

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The precise molecular identity of the renal ATP-regulated secretory K^+ channel is still a matter of some controversy. The inwardly rectifying K^+ channel, Kir1.1 (ROMK) appears to form the pore of the channel, and mutations in Kir1.1 are responsible for Bartter syndrome. The native channel is sensitive to inhibition by the sulfonylurea glibenclamide, and it has been proposed that an accessory protein is required to confer glibenclamide sensitivity to Kir1.1. Several recent studies have suggested that the native channel is composed of the splice variant Kir1.1b (ROMK2) and the sulfonylurea receptor isoform SUR2B and that there is a direct physical interaction between these subunits. In this study, we have monitored the interaction between Kir1.1b and SUR2B. We find that SUR2B reaches the plasma membrane when coexpressed with Kir6.1 or Kir6.2 but not when coexpressed with Kir1.1b. Furthermore, we find that Kir1.1b exhibits an intrinsic sensitivity to inhibition by glibenclamide with an affinity similar to the native channel. These results demonstrate that SUR2B does not traffic to the membrane in the presence of Kir1.1b and is not required to confer glibenclamide sensitivity to Kir1.1b. This has important implications for the presumed structure of the renal ATP-regulated secretory K^+ channel.

The principal mechanism of K^+ excretion by the body is the selective secretion of K^+ by the kidney. This is achieved by the renal cortical collecting duct principal cells where K^+ is secreted into the urine through the apical ATP-regulated secretory K^+ channel (1, 2). Studies of cloned channels have shown that the inwardly rectifying K^+ channel Kir1.1 (ROMK) is expressed in the apical membrane of these cells (3) and possesses very similar conductive and kinetic properties to the native channel (4, 5). Further evidence to support the role of this channel comes from genetically inherited mutations in Kir1.1, which are responsible for Type II Bartter syndrome (6). However, the native channel is inhibited by the sulfonylurea drug glibenclamide (7), and several studies on Kir1.1 have suggested that this property is lacking in the cloned channel (8, 9). This apparent difference in pharmacology has led to the assumption that Kir1.1 associates with an additional "regulatory" subunit *in vivo* that would confer sensitivity to glibenclamide (1).

In an attempt to identify this missing subunit, comparisons have been drawn with the classic ATP-sensitive (K_{ATP}) potassium channel that regulates insulin secretion from the pancreatic β-cell and that is also inhibited by glibenclamide. The β-cell K_{ATP} channel consists of an inwardly rectifying K^+ channel (Kir6.2) that physically associates with the sulfonylurea receptor, SUR1. This regulatory subunit confers the stimulatory effect of nucleotides as well as high affinity inhibition by glibenclamide (10). A similar K_{ATP} channel found in the heart is also comprised of Kir6.2 and a related sulfonylurea receptor, SUR2A (11).

Both SUR1 and SUR2A are members of the ATP-binding cassette (ABC)[1] superfamily of transporters and are related to the cystic fibrosis conductance regulator (CFTR), which is also inhibited by glibenclamide (12). It has therefore been proposed that the missing regulatory subunit may be a renal ABC transporter that couples to Kir1.1 (1). Both CFTR and an SUR2A splice variant (SUR2B) are expressed in the cortical collecting duct (9, 13–15), and studies have shown that both of these ABC transporters appear to confer glibenclamide sensitivity to Kir1.1 when coexpressed in *Xenopus* oocytes (8, 9, 16). Both SUR2B and CFTR have therefore been proposed as potential candidates for the "missing regulatory subunit." Recent studies on the interaction between Kir1.1 and SUR2B have proposed that it is an N-terminal splice variant of Kir1.1 referred to as Kir1.1b (ROMK2) that physically associates with SUR2B to form the native channel (9). Another recent study has suggested that this functional and physical association is governed by a motif in the intracellular N terminus of Kir1.1 (17).

Studies on the assembly of classic K_{ATP} channels formed by members of the Kir6.0 subfamily coexpressed with a sulfonylurea receptor have shown that the correct stoichiometry of assembly is achieved by the presence of endoplasmic reticulum (ER) retention signals on the intracellular domains of both subunits (18, 19). These ER retention signals cause both subunits to be retained within the cell unless correctly coassembled into an octameric (4 + 4) complex. The ER retention signal is an "RKR" motif located on the distal C terminus of Kir6.2 and Kir6.1, whereas on SUR1 it is adjacent to the first nucleotide-binding fold. SUR2A contains a similar although not identical (RKQ) motif, but this also functions as a retention signal, causing it to be retained within the cell unless coexpressed with either Kir6.1 or Kir6.2.[2] SUR2B differs from SUR2A only in the last 42 amino acids (20) and thus also contains an ER retention signal. However, the role of this motif

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[1] The abbreviations used are: ABC, ATP-binding cassette; HA, hemagglutinin; ER, endoplasmic reticulum; CFTR, cystic fibrosis conductance regulator; SUR, sulfonylurea receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate.

[2] Blanche Schwappach, personal communication.

in the trafficking of SUR2B has not been assessed. Therefore, the recent reports that SUR2B associates with Kir1.1b to confer glibenclamide sensitivity (9, 17) suggest either that SUR2B is capable of independent trafficking to the membrane or that Kir1.1b physically associates with SUR2B to form functional complexes that can then traffic to the plasma membrane.

In this study, we assessed the functional interaction between Kir1.1b and SUR2B coexpressed in *Xenopus* oocytes. In addition to measuring channel activity, we also monitored the surface expression of SUR2B using the chemiluminescent antibody detection method developed by Zerangue *et al.* (18) and Schwappach *et al.* (19). We found that the ER retention motif on SUR2B causes it to be retained within the cell and that it does not traffic to the membrane in the presence of Kir1.1b. Furthermore, we also found that Kir1.1b can be directly inhibited by glibenclamide in the absence of SUR2B and that detection of this inhibitory effect is dependent upon the methods used to study channel activity. These observations therefore demonstrate that Kir1.1b has an intrinsic sensitivity to glibenclamide that does not depend upon the presence of SUR2B. These results have important implications for the presumed structure of the native ATP-regulated secretory K^+ channel.

MATERIALS AND METHODS

Molecular Biology

Rat Kir1.1a, Kir4.1, and SUR2B and mouse Kir2.1, Kir6.1, and Kir6.2 were subcloned in the oocyte expression vector pBF. For surface expression studies, rat SUR2B had the hemagglutinin (HA) epitope introduced at the same site as in SUR1 (18, 19) (a gift from Dr. B. Schwappach, Heidelberg, Germany). N-terminal deletion of the first 19 amino acids of Kir1.1a to generate Kir1.1b (ROMK2) (21) was performed by PCR. Site-directed mutagenesis was performed using the QuikChange XL protocol (Stratagene, La Jolla, CA) to engineer $SUR2B_{R\text{-}K}HA$. Capped mRNAs were synthesized *in vitro* by using the T7 or SP6 mMESSAGE mMACHINE kit (Ambion, Austin, TX).

Isolation of Oocytes and Injection of cRNA

Xenopus laevis oocytes were prepared and injected as described (22). Defolliculated oocytes were injected with various cRNA combinations. For each potassium channel, 1 ng of cRNA was used, whereas 20 ng of cRNA was used for SUR2B. Injected oocytes were kept in modified Barth's saline (in mM: 88, NaCl; 1, KCl; 2.4, $NaHCO_3$; 0.3, $Ca(NO_3)_2$; 0.41, $CaCl_2$; 0.82, $MgSO_4$; 15, HEPES; adjusted to pH 7.6 with Tris).

Electrophysiology

Two-electrode Voltage Clamp—Using the pBF expression vectors, we observed near maximal expression of Kir6.2/SUR2B currents after 36–48 h (not shown). Thus, all oocytes were studied 2 days after injection using the two-electrode voltage clamp technique as described previously (22). Using protocols similar to those described by Tanemoto *et al.* (9), oocytes were routinely clamped at a holding potential of −80 mV and intermittently pulsed over the range of −160 to +40 mV in 20-mV steps, each lasting 400 ms. Reported K^+ current values refer to those measured at a holding potential of −140 mV during the last 100 ms of a pulse. Glibenclamide (Sigma) was prepared as a 200 mM stock solution in Me_2SO. The glibenclamide-sensitive current was determined by subtracting the corresponding value measured in the presence of 0.2 mM glibenclamide from that measured prior to the application of glibenclamide in a 1 mM external KCl solution (in mM: 96, NaCl; 1, KCl; 1.8, $CaCl_2$; 1, $MgCl_2$; 5, HEPES; adjusted to pH 7.4 with Tris). In all batches of oocytes tested, coexpression of Kir6.2 or Kir6.1 with SUR2B resulted in typical K_{ATP} K^+ currents activated by exposure to 3 mM sodium azide. Under "Results," data are given as mean values ± S.E. n indicates the number of oocytes, and N indicates the number of different batches of oocytes used; significance was evaluated by the appropriate version of Student's t test.

Macropatch Recording—For macroscopic recordings from giant excised inside-out patches, the patch pipettes were pulled from thick-walled borosilicate glass and had resistances of 250–500 kiloohms when filled with pipette solution. Macroscopic currents were recorded at a holding potential of 0 mV and at 20–24 °C (23). Currents were evoked by repetitive 3-s voltage ramps from −110 mV to +100 mV and recorded as described previously (23). The pipette (external) solution contained (in mM): 140, KCl; 1.2, $MgCl_2$; 2.6, $CaCl_2$; 10, HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained (in mM): 110, KCl; 1.4, $MgCl_2$; 10, EGTA; 10, HEPES (pH 7.5 with KOH; final $[K^+]$ ~115 mM). 0.1 mM MgATP was added to the bath solution to prevent rundown of channel activity. The pH of the solution was readjusted after addition of the MgATP and glibenclamide. 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. The slope conductance was measured by fitting a straight line to the current-voltage relation between −20 mV and −100 mV. Conductance was measured from an average of five consecutive ramps in each solution. In ~50% of patches, currents ran down with time in a linear fashion. To control for rundown, a straight line was fitted to the decay of the slope conductance in control solution and extrapolated to the same time point at which the slope conductance was measured in the presence of drug. This value was then taken as the control slope conductance level. Responses to glibenclamide were expressed relative to the conductance measured in control solution before the application of glibenclamide. The concentration-response curve was constructed by expressing the conductance in the presence of glibenclamide (G) as a fraction of that in control solution (G_C).

Surface Labeling of Oocytes

Experiments were essentially performed as recently described (18, 22) using 1 μg/ml rat monoclonal anti-HA antibody (clone 3F10, Roche Molecular Biochemicals) as primary antibody and 2 μg/ml peroxidase-conjugated affinity-purified $F(ab')_2$ fragment goat anti-rat IgG antibody (Jackson ImmunoResearch) as secondary antibody. Surface expression is expressed in 1000 relative light units/15 s/oocyte.

RESULTS

Inhibition of Kir1.1 Channels by Glibenclamide Is Time-dependent—We first sought to reproduce the reported functional interaction between Kir1.1b and SUR2B (9, 17). We therefore examined the effects of glibenclamide (0.2 mM) on Kir1.1b expressed in *Xenopus* oocytes either with or without coexpression of SUR2B. We measured macroscopic whole cell currents by two-electrode voltage clamp before and after the application of 0.2 mM glibenclamide (Fig. 1*A*). A low external $[K^+]$ bath solution was used as this has been reported to be required for optimal inhibition by glibenclamide (9, 17). Fig. 1*A* shows a continuous whole cell current recording at a holding potential of −80 mV made from an oocyte expressing Kir1.1b alone. After 2 min, glibenclamide has only a small inhibitory effect. The remaining currents could be inhibited by 5 mM Ba^{2+}, indicating that they are K^+ selective. Additional voltage step protocols were performed at the times indicated by *asterisks*. These are shown in Fig. 1*B*, which demonstrates that similar results were obtained in oocytes coexpressing Kir1.1b and SUR2B. Fig. 1*C* summarizes the inhibitory effect of 0.2 mM glibenclamide after 2 min on Ba^{2+}-sensitive K^+ currents recorded from both Kir1.1a and Kir1.1b in the presence and absence of coexpressed SUR2B. As a control, Kir6.2 was coexpressed with SUR2B to form functional glibenclamide-sensitive K_{ATP} channels. The results show that after 2 min, 0.2 mM glibenclamide inhibited Kir6.2/SUR2B currents by 96.0 ± 0.4% (n = 7; N = 1; p < 0.001), whereas Kir1.1a and Kir1.1b currents were only inhibited by 13.0 ± 3.1% (n = 21; N = 3; p < 0.001) and 10.3 ± 1.7% (n = 21; N = 3; p < 0.001), respectively. Coexpression of SUR2B did not alter the glibenclamide sensitivity of either Kir1.1a or Kir1.1b since 0.2 mM glibenclamide inhibited Kir1.1a/SUR2B and Kir1.1b/SUR2B currents by 12.5 ± 1.9% (n = 21; N = 3; p < 0.001) and 12.1 ± 2.7% (n = 21; N = 3; p < 0.001), respectively.

Given the limited inhibition of Kir1.1b/SUR2B currents by 0.2 mM glibenclamide after 2 min, we next examined the effects of longer exposure to glibenclamide. Fig. 2*A* shows that significant glibenclamide inhibition of Kir1.1b currents could be observed after 10 min. However, this did not depend on the presence of coexpressed SUR2B. Fig. 2 (*B* and *C*) shows that Kir1.1b currents exhibited similar levels of inhibition in the

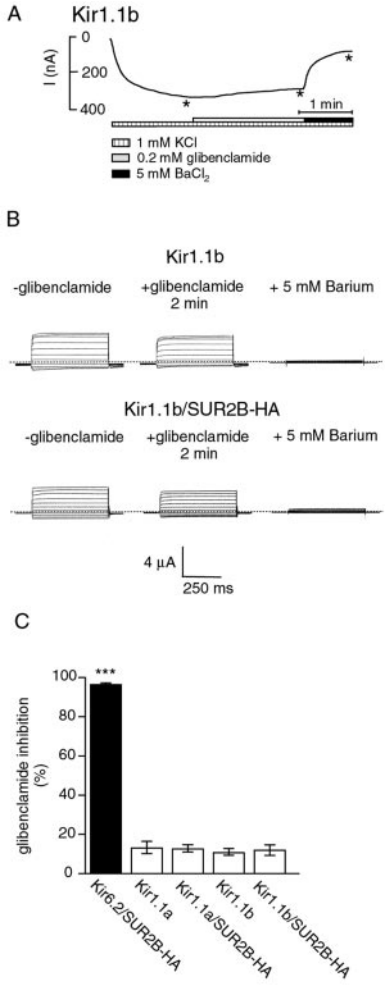

FIG. 1. **Limited inhibition of Kir1.1/SUR2B currents by glibenclamide after 2 min.** *A*, whole cell currents (*I*) recorded at a holding potential of −80 mV from an oocyte expressing Kir1.1b. The oocyte was bathed in a 1 mM KCl solution, and once maximal currents were reached, 0.2 mM glibenclamide was added. 2 min after glibenclamide application, 5 mM $BaCl_2$ was added, which completely inhibited the inward K^+ currents. Voltage step protocols were performed at the times indicated by *asterisks* and are shown below. *B*, representative whole cell current families from oocytes either expressing Kir1.1b or coexpressing Kir1.1b and SUR2B-HA (see "Materials and Methods"). The *dotted line* represents the zero current level. *C*, average inward current inhibition 2 min after addition of 0.2 mM glibenclamide. Currents were recorded at −140 mV for Kir1.1a, Kir1.1a/SUR2B-HA, Kir1.1b, and Kir1.1b/SUR2B-HA oocytes (*open bars*) and for Kir6.2/SUR2B oocytes (*closed bars*). The % inhibition of K^+ currents by glibenclamide was normalized to the initial currents prior to glibenclamide addition. For Kir6.2/SUR2B-HA, $n = 7$, and for all other groups, $n = 21$.

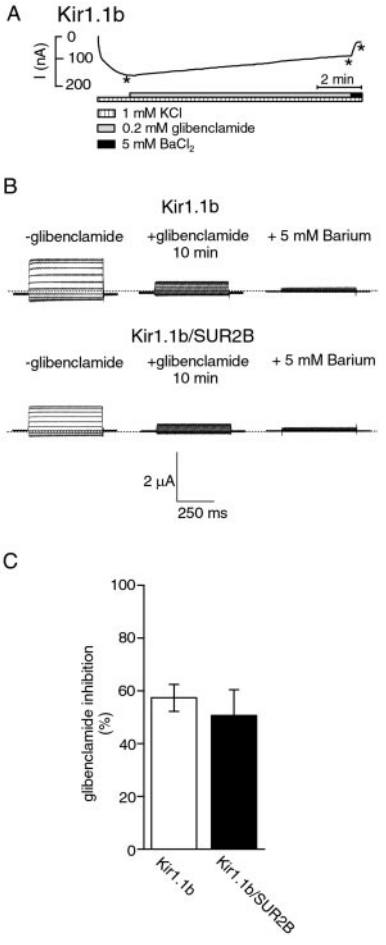

FIG. 2. **Continued exposure to 0.2 mM glibenclamide inhibits Kir1.1b currents.** *A*, whole cell currents (*I*) recorded from an oocyte expressing Kir1.1b as described in the legend for Fig. 1. 10 min after glibenclamide application, 5 mM $BaCl_2$ was added. Voltage step protocols were performed at times indicated by *asterisks*. *B*, representative whole cell current families from oocytes either expressing Kir1.1b or coexpressing Kir1.1b/SUR2B. Experiments were performed as described in the legend for Fig. 1 (*panel B*), but this time, glibenclamide (0.2 mM) was applied for 10 min. *C*, the % inhibition by glibenclamide normalized for Kir1.1b (*open bars*) and Kir1.1b/SUR2B oocytes (*closed bars*) as described in the legend for Fig. 1 (*panel C*). Glibenclamide inhibited the K^+ currents in both groups with similar potency ($n = 7$ for both groups).

absence of coexpressed SUR2B. The application of 0.2 mM glibenclamide for 10 min inhibited Kir1.1b and Kir1.1b/SUR2B currents by 58.1 ± 5.1% ($n = 7$; $N = 1$; $p < 0.001$) and 54.7 ± 6.4% ($n = 7$; $N = 1$; $p < 0.001$), respectively (Fig. 2*C*). The block of Kir1.1b currents by glibenclamide was not voltage-dependent since the currents showed similar levels of inhibition when recorded at +40 mV: Kir1.1b and Kir1.1b/SUR2B currents were inhibited by 57.7 ± 5.9% ($n = 7$; $N = 1$; $p < 0.001$) and 53.9 ± 5.4% ($n = 7$; $N = 1$; $p < 0.001$), respectively.

These results clearly demonstrate that Kir1.1b possesses an intrinsic sensitivity to high concentrations of glibenclamide which can only be observed after long periods of exposure. They also demonstrate that coexpression of SUR2B does not affect the inhibition of Kir1.1a or Kir1.1b by 0.2 mM glibenclamide.

SUR2B Does Not Reach the Plasma Membrane in the Presence of Kir1.1b—Given the lack of effect of SUR2B coexpression upon the glibenclamide sensitivity of Kir1.1b we next examined the role of the ER retention signal in SUR2B and whether SUR2B actually reaches the plasma membrane in the presence of Kir1.1b. To detect the surface expression of SUR2B, we used a variant that had an HA antigen epitope engineered into an extracellular loop (SUR2B-HA) (19). This epitope permits detection of SUR2B in the plasma membrane by chemiluminescent detection of anti-HA antibody binding. Fig. 3 shows that like SUR1 and SUR2A, SUR2B-HA does not reach the plasma membrane when expressed by itself. However, when coexpressed with Kir6.2, surface labeling could be detected, thus indicating the presence of SUR2B in the surface plasma membrane of the oocytes. Similar surface expression of SUR2B-HA could also be detected when coexpressed with Kir6.1 (not shown). This also correlated with the formation of functional K_{ATP} channels in these oocytes (not shown). However, Fig. 3 also demonstrates that no surface expression could be detected when SUR2B-HA was coexpressed with Kir1.1b, Kir2.1, or Kir4.1. These groups all expressed K^+-selective currents about 10-fold larger than the Kir6.2/SUR2B-HA currents (not shown). Assuming a similar stoichiometry of assembly between

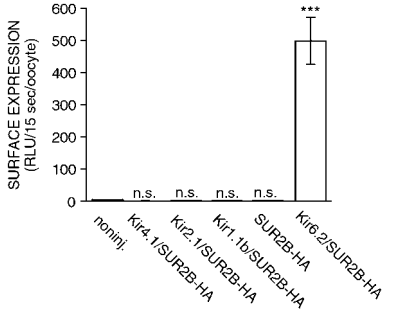

FIG. 3. **SUR2B does not traffic to the membrane in the presence of Kir1.1b.** A chemiluminescence antibody detection assay was used to measure the surface expression of SU2B-HA in oocytes expressing SUR2B-HA alone or coexpressing SUR2B-HA with Kir6.2, Kir4.1, Kir2.1, or Kir1.1b. Except when coexpressed with Kir6.2, surface labeling of the above groups was similar to non-injected (*noninj*) oocytes. Surface expression is expressed as relative light units/15 s/oocyte. For all groups, $n = 10$. *n.s.*, not significant.

Kir1.1b and SUR2B-HA as found between Kir6.2 and SUR2A (19), we would expect a larger surface labeling signal in the Kir1.1b/SUR2B-HA oocytes as compared with the Kir6.2/SUR2B-HA oocytes. Instead, the surface labeling in the Kir1.1b/SUR2B-HA oocytes was not different from H_2O-injected controls or SUR2B-HA coexpressed with Kir2.1 or Kir4.1. This indicates that virtually no SUR2B-HA is detectable in the surface membrane of these oocytes and is consistent with previous studies demonstrating the specificity of interaction between Kir6.0 subunits and sulfonylurea receptors (18, 19, 24).

Lack of Interaction between Kir1.1b and SUR2B When Coexpressed in the Membrane—Given that SUR2B is unlikely to confer glibenclamide sensitivity to Kir1.1b if it is not present in the plasma membrane, we next examined whether SUR2B could alter the glibenclamide sensitivity of Kir1.1b when both subunits were present in the plasma membrane at the same time. We therefore confirmed that the RKQ ER retention signal in SUR2B was responsible for its lack of surface expression in *Xenopus* oocytes. Fig. 4*A* shows that coexpression with Kir1.1b does not permit trafficking of SUR2B-HA to the plasma membrane but that when the ER retention signal is mutated to "KKQ" ($SUR2B_{R\text{-}K}$-HA), the mutant $SUR2B_{R\text{-}K}$-HA subunits traffic independently to the plasma membrane. Fig. 4*A* also shows that coexpression of Kir1.1b with $SUR2B_{R\text{-}K}$-HA did not affect trafficking of $SUR2B_{R\text{-}K}$-HA to the plasma membrane.

We therefore examined the glibenclamide sensitivity of the Kir1.1b channels coexpressed with $SUR2B_{R\text{-}K}$-HA where both subunits were clearly present in the plasma membrane at the same time. Fig. 4*B* shows that a 10-min exposure to 0.2 mM glibenclamide inhibits Kir1.1b/$SUR2B_{R\text{-}K}$-HA currents by almost 50%. However, Fig. 4*C* shows that this degree of inhibition is no different from that seen when Kir1.1b is expressed alone (or with SUR2B-HA, as shown in Fig. 2*C*). 0.2 mM glibenclamide inhibited Kir1.1b and Kir1.1b/$SUR2B_{R\text{-}K}$-HA currents by 54.7 ± 6.4% ($n = 7$; $N = 1$) and 50.7 ± 10.1% ($n = 21$; $N = 3$), respectively.

Fast and Reversible Block of Kir1.1b by Glibenclamide in Giant Excised Patches—One explanation for the relatively slow inhibition of Kir1.1 currents observed in whole cell two-electrode voltage clamp recordings is that inhibition occurs via an intracellular site. It would therefore take longer for high concentrations of glibenclamide to equilibrate across the vitelline and plasma membranes of oocytes when applied extracellularly. By contrast, Kir6.2/SUR currents are sensitive to inhibition by nanomolar concentrations of glibenclamide, and thus, rapid inhibition can be observed even when low concentrations

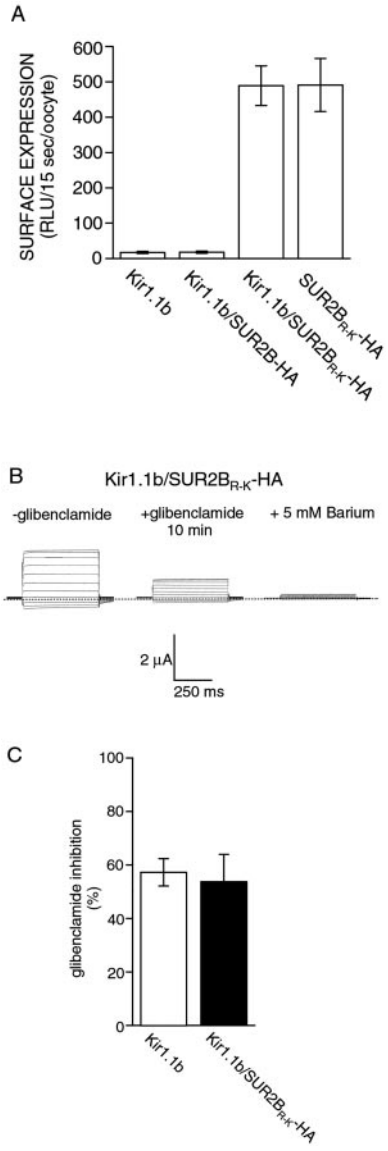

FIG. 4. **Lack of interaction between Kir1.1b and SUR2B in the plasma membrane.** *A*, surface labeling was low in oocytes either expressing Kir1.1b alone or coexpressing Kir1.1b with SUR2B-HA. By contrast, significant surface labeling is observed in oocytes expressing either mutant $SUR2B_{R\text{-}K}$-HA alone or coexpressing $SUR2B_{R\text{-}K}$-HA and Kir1.1b ($n = 10$). *B*, representative whole cell current families from oocytes coexpressing either Kir1.1b and $SUR2B_{R\text{-}K}$-HA (see Fig. 1*B*). 0.2 mM glibenclamide was applied for 10 min. *C*, glibenclamide inhibition of Kir1.1b (*open bars*) and Kir1.1b/$SUR2B_{R\text{-}K}$-HA currents (*solid bars*), $n = 7$.

of drug are applied (10). This time dependence of inhibition may also explain the variability in the reported effects of glibenclamide on Kir1.1, depending on how long after the application inhibition is measured. We therefore used giant inside-out patches excised from *Xenopus* oocytes expressing Kir1.1b to examine the inhibitory effect of glibenclamide.

Fig. 5*A* shows that K^+ currents recorded from inside-out macropatches excised from oocytes expressing Kir1.1b are inhibited directly by glibenclamide. The inhibition by glibenclamide is concentration-dependent, and the mean data (Fig. 5*B*) suggest a half-maximal inhibition around 150–200 µM glibenclamide. The observed rundown of channel activity is characteristic of Kir1.1b behavior in excised patches (21). 50 µM glibenclamide inhibits 28.2 ± 4.5% ($n = 6$; $N = 2$), 0.2 mM inhibits 54.1 ± 3.9% ($n = 15$; $N = 2$), and 0.5 mM inhibits 74.9 ± 8.1% ($n = 5$; $N = 2$) of the current in control (drug-free)

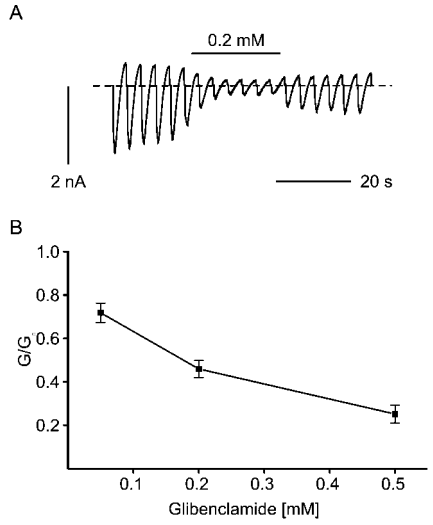

FIG. 5. **Effect of glibenclamide on Kir1.1b currents in excised macropatches.** *A*, macroscopic currents recorded from inside-out macropatches in response to a series of voltage ramps from −110 mV to +100 mV from oocytes expressing Kir1.1b alone. Glibenclamide was added to the intracellular solution as indicated by the bar. *B*, mean macroscopic slope conductance in the presence of glibenclamide (G) expressed as a fraction of the mean slope conductance in control solution (G_C). *Straight lines* were used to connect the points at 50 μM (n = 6), 200 μM (n = 15), and 500 μM (n = 5) glibenclamide.

solution. These results clearly show that high concentrations of glibenclamide can cause significant inhibition of Kir1.1b currents. Furthermore, the effect is rapid (within seconds) and reversible.

DISCUSSION

In this study, we have demonstrated that Kir1.1b possesses an intrinsic sensitivity to inhibition by glibenclamide that is similar to the native renal ATP-regulated secretory K^+ channel. Our results indicate that the reported variability in sensitivity of Kir1.1b to glibenclamide is probably due to differences in the experimental protocols used to study channel activity. Furthermore, we demonstrate that an ER retention signal prevents SUR2B from trafficking to the plasma membrane when coexpressed with Kir1.1b, and therefore, SUR2B cannot confer glibenclamide sensitivity to Kir1.1b. We also show that even if SUR2B is mutated to traffic to the plasma membrane, it does not influence the intrinsic glibenclamide sensitivity of Kir1.1b. Thus, contrary to recent reports, our results demonstrate that SUR2B is unlikely to be a component of the native renal secretory K^+ channel and that Kir1.1 does not require an accessory subunit to be inhibited by glibenclamide.

One of the principal aims of this study was to investigate recent reports that the renal ATP-regulated secretory K^+ channel is comprised of Kir1.1b and SUR2B. The studies by Tanemoto *et al.* (9) and Dong *et al.* (17) have reported a physical association between these two subunits as evidenced by coimmunoprecipitation of *in vitro* translated Kir1.1b and SUR2B. Their studies also reported that Kir1.1b exhibits no intrinsic sensitivity to inhibition by glibenclamide but that coexpression with SUR2B "restores" sensitivity of the channel to 0.2 mM glibenclamide. However, there are certain difficulties associated with the hypothesis that SUR2B associates with Kir1.1b to form the native channel. Firstly, only very high concentrations of glibenclamide have been shown to inhibit Kir1.1b/SUR2B channels (9, 17), which does not correlate with the high affinity interaction of glibenclamide with SUR2B (10, 25). Secondly, studies with other sulfonylurea receptors suggest that SUR2B is unlikely to traffic to the plasma membrane in the presence of Kir1.1 (18, 19).

Low Affinity Versus High Affinity Effects of Glibenclamide—It is essential to distinguish between the reported effects of glibenclamide at low (nM) concentrations and its effects at high (from μM to mM) concentrations. Although high affinity inhibition of the classic K_{ATP} channels (Kir6.0/SUR) by nanomolar concentrations of glibenclamide is one of the hallmark features of these channels (10, 25), it is well known that high concentrations of glibenclamide can interact with other membrane proteins. Examples of its wide-ranging influences include effects on volume-sensitive anion channels (26), outwardly rectifying chloride channels (27), voltage-gated K^+ channels (28), and epithelial Na^+ channels (29). The response of a channel to glibenclamide does not therefore automatically implicate the involvement of a classic sulfonylurea receptor subunit.

The fact that SUR subunits are ABC transporters has also led to the suggestion that glibenclamide may interact with other ABC transporters. Effects of glibenclamide have been observed on both CFTR (12) and P-glycoprotein function (30), but again, only at relatively high concentrations when compared with the nanomolar affinity of the sulfonylurea receptors. Using the inhibitory effect of high concentrations of glibenclamide as a hallmark for correlating cloned channel behavior with that of native channels is therefore potentially confusing. Indeed, we and others have shown previously that high concentrations of glibenclamide and other sulfonylureas such as tolbutamide can inhibit Kir6.2 channel activity directly and that it is essential to take this low affinity inhibition into account when interpreting the mechanism of K_{ATP} channel block by sulfonylureas (23, 31, 32). Therefore, it is perhaps not surprising that other related channels such as Kir1.1 also exhibit a similar intrinsic sensitivity to high concentrations of glibenclamide.

Variability in Response of Kir1.1 to Glibenclamide—The original study that reported the cloning of Kir1.1b (ROMK2) demonstrated a clear but variable inhibition of Kir1.1b currents by 0.2 mM glibenclamide (21). Subsequent studies have also reported either limited or no inhibition of Kir1.1b currents by high concentrations of glibenclamide (9, 16, 17). Our results suggest that this variability depends on the experimental methods and parameters used to study Kir1.1. In most cases, whole cell macroscopic currents are recorded by two-electrode voltage clamp (9, 17, 21). Sulfonylureas inhibit K_{ATP} channel activity from the intracellular surface (33). It is clear from the results shown in Fig. 5 that 0.2 mM glibenclamide can inhibit Kir1.1b channel activity rapidly and reversibly when applied to the intracellular surface of an excised patch. However, when measuring whole cell currents by two-electrode voltage clamp, it may take significantly longer for high concentrations of glibenclamide to equilibrate across the plasma membrane of the oocytes when applied extracellularly. This might account for the time dependence of inhibition that we observe and for the variability in the reported effects of glibenclamide, depending on how long after application the channel activity is measured.

Although we have not calculated a full dose response curve, Fig. 5*B* illustrates that 0.2 mM glibenclamide inhibits channel activity by slightly greater than 50%. This suggests that half-maximal inhibition occurs in the 150–200 μM range. This value is remarkably similar to that reported for the inhibition of the native channel (150 μM) (7).

Trafficking of SUR2B to the Plasma Membrane—This study shows a lack of functional interaction between Kir1.1b and SUR2B. However, previous studies have reported that Kir1.1b and SUR2B can physically associate when cotranslated *in vitro* (9, 17). This suggests that Kir1.1b may nevertheless be capable of associating with SUR2B to form Kir1.1b/SUR2B channel

complexes *in vivo*. Although the role of the ER retention signals in SUR1 and SUR2A has been well studied (18, 19), the role of the RKQ motif in SUR2B has not been analyzed. Our results demonstrate that this motif causes SUR2B to be retained inside the cell unless coexpressed with Kir6.2. We also found that Kir1.1b, Kir2.1, and Kir4.1 were unable to promote trafficking of SUR2B to the plasma membrane. Such results are consistent with previous reports that only Kir6.1 and Kir6.2 can physically associate with SUR1 or SUR2A (18, 19, 24).

We therefore conclude that SUR2B does not appear in the plasma membrane when coexpressed with Kir1.1b. Thus, it is unlikely that SUR2B can functionally couple glibenclamide sensitivity to Kir1.1b when they are coexpressed in *Xenopus* oocytes. However, by mutating the ER retention signal in SUR2B, we were able to express both Kir1.1b and SUR2B in the plasma membrane at the same time. Nevertheless, even when both proteins were present together, SUR2B still had no effect on the intrinsic glibenclamide sensitivity of Kir1.1b. This indicates that the lack of functional coupling *in vivo* is probably due to a lack of physical association. This is in contrast to the reported physical association of Kir1.1b and SUR2B analyzed by coimmunoprecipitation of *in vitro* translated proteins (9, 17). We are unable to reconcile these differences other than to suggest that the interactions observed *in vitro* may not reflect the behavior of these integral membrane proteins *in vivo*.

What Is the Molecular Identity of the Native Renal Secretory K^+ Channel?—Our results do not support a role for SUR2B as a component of the renal secretory K^+ channel. Furthermore, our results indicate that there is no requirement for an additional subunit to confer glibenclamide sensitivity to Kir1.1b. Thus, the physiological role of SUR2B in the cortical collecting duct principal cell remains unclear. However, Kir6.1 expression has been reported in these cells (34), and it remains possible that SUR2B and Kir6.1 associate to form a classic K_{ATP} channel, although to date, no K_{ATP}-like currents have been recorded in these cells.

Many different studies have shown that Kir1.1b has conductive and kinetic properties that are very similar to those of the native channel (5, 21), and this study now demonstrates that another distinguishing characteristic of the native channel (*i.e.* glibenclamide sensitivity) is intrinsic to Kir1.1b. It is thus tempting to speculate that the native channel is simply Kir1.1 (or a splice variant) and that no additional subunits are required. However, two principal concerns remain regarding the identity of the channel, and we have not directly addressed these issues in this study. Firstly, is the ATP regulation of Kir1.1 identical to that of the native channel? Secondly, what is the role of CFTR?

The regulation of the channel by ATP is complex. Low levels of MgATP are required to maintain the activity of the native channel, but higher (mM) concentrations inhibit channel activity (1). However, there is some controversy regarding the ATP sensitivity of the cloned channel. It has been reported that Kir1.1b channels can be inhibited by ATP (35) but that Kir1.1a is insensitive to ATP (4). Some of these differences may be accounted for by the fact that Kir1.1 channels can also be activated by PIP_2 (36) with a requirement for MgATP in the generation of PIP_2 by lipid kinases. The effects of ATP therefore can vary depending upon the presence or absence of different kinases under different metabolic and experimental conditions.

The effect of CFTR coexpression on Kir1.1 activity is equally complex. CFTR coexpression has been reported to influence a variety of Kir1.1 properties including its single channel conductance (8), sensitivity to ATP and glibenclamide (8, 16), and also trafficking (37). Based upon our results, it is now clear that an accessory subunit is not required to confer glibenclamide sensitivity upon Kir1.1. However, whether there is a more complex role for CFTR in influencing Kir1.1 channel activity *in vivo* remains to be determined.

REFERENCES

1. Wang, W., Hebert, S. C., and Giebisch, G. (1997) *Annu. Rev. Physiol.* **59,** 413–436
2. Giebisch, G. (2001) *Kidney Int.* **60,** 436–445
3. Mennitt, P. A., Wade, J. B., Ecelbarger, C. A., Palmer, L. G., and Frindt, G. (1997) *J. Am. Soc. Nephrol.* **8,** 1823–1830
4. Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V., and Hebert, S. C. (1993) *Nature* **362,** 31–38
5. Palmer, L. G., Choe, H., and Frindt, G. (1997) *Am. J. Physiol.* (*Cell Physiol.*) **273,** F404–F410
6. Simon, D. B., Karet, F. E., Rodriguez-Soriano, J., Hamdan, J. H., DiPietro, A, Trachtman, H, Sanjad, S. A., and Lifton, R. P. (1996) *Nat. Genet.* **14,** 152–156
7. Wang, T., Wang, W. H., Klein-Robbenhaar, G., and Giebisch, G. (1995) *Ren. Physiol. Biochem.* **18,** 169–182
8. Ruknudin, A., Schulze, D. H., Sullivan, S. K., Lederer, W. J., and Welling, P. A. (1998) *J. Biol. Chem.* **273,** 14165–14171
9. Tanemoto, M., Vanoye, C. G., Dong, K., Welch, R., Abe, T., Hebert, S. C., and Xu, J. Z. (2000) *Am. J. Physiol.* (*Renal Physiol.*) **278,** F659–F666
10. Ashcroft, F. M., and Gribble, F. M. (1999) *Diabetologia* **42,** 903–919
11. Inagaki, N., Gonoi, T., Clement, J. P., Wang, C. Z., Aguilar Bryan, L., Bryan, J., and Seino, S. (1996) *Neuron* **16,** 1011–1017
12. Sheppard, D. N., and Welsh, M. J. (1993) *Ann. N. Y. Acad. Sci.* **707,** 275–284
13. Devuyst, O., Burrow, C. R., Schwiebert, E. M., Guggino, W. B., and Wilson, P. D. (1996) *Am. J. Physiol.* (*Renal Physiol.*) **271,** F723–F735
14. Morales, M. M., Carroll, T. P., Morita, T., Schwiebert, E. M., Devuyst, O., Wilson, P. D., Lopes, A. G., Stanton, B. A., Dietz, H. C., Cutting, G. R., and Guggino, W. B. (1996) *Am. J. Physiol.* (*Renal Physiol.*) **270,** F1038–F1048
15. Beesley, A. H., Qureshi, I. Z., Giesberts, A. N., Parker, A. J., and White, S. J. (1999) *Pflügers Arch.* **438,** 1–7
16. McNicholas, C. M., Guggino, W. B., Schwiebert, E. M., Hebert, S. C., Giebisch, G., and Egan, M. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8083–8088
17. Dong, K., Xu, J., Vanoye, C. G., Welch, R., MacGregor, G. G., Giebisch, G., and Hebert, S. C. (2001) *J. Biol. Chem.* **276,** 44347–44353
18. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* **22,** 537–548
19. Schwappach, B., Zerangue, N., Jan, Y. N., and Jan, L. Y. (2000) *Neuron* **26,** 155–167
20. Isomoto, S., Kondo, C., Yamada, M., Matsumoto, S., Higashiguchi, O., Horio, Y., Matsuzawa, Y., and Kurachi, Y. (1996) *J. Biol. Chem.* **271,** 24321–24324
21. Zhou, H., Tate, S., and Palmer, L. G. (1994) *Am. J. Physiol.* (*Cell Physiol.*) **266,** C809–C824
22. Konstas, A. A., Bielfeld-Ackermann, A., and Korbmacher, C. (2001) *Pflügers Arch* **442,** 752–761
23. Gribble, F. M., Ashfield, R., Ammala, C., and Ashcroft, F. M. (1997) *J. Physiol.* (*Lond.*) **498,** 87–98
24. Clement, J. P., Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguilar-Bryan, L., and Bryan, J. (1997) *Neuron* **18,** 827–838
25. Russ, U., Hambrook, A., Artunc, F., Loffler-Walz, C., Horio, Y., Kurachi, Y., and Quast, U. (1999) *Mol. Pharmacol.* **55,** 955–961
26. Best, L., and Benington, S. (1998) *Br. J. Pharmacol.* **125,** 874–878
27. Volk, T., Rabe, A., and Korbmacher, C. (1995) *Cell. Physiol. Biochem.* **5,** 222–231
28. Yao, X., Chang, A. Y., Boulpaep, E. L., Segal, A. S., and Desir, G. V. (1996) *J. Clin. Invest.* **97,** 2525–2533
29. Chrabi, A., and Horisberger, J. D. (1999) *J. Pharmacol. Exp. Ther.* **290,** 341–347
30. Golstein, P. E., Boom, A., van Geffel, J., Jacobs, P., Masereel, B., and Beauwens, R. (1999) *Pflügers Arch.* **437,** 652–660
31. Gros, L., Virsolvy, A., Salazar, G., Bataille, D., and Blache, P. (1999) *Biochem. Biophys. Res. Commun.* **257,** 766–770
32. Gribble, F. M., Tucker, S. J., Seino, S., and Ashcroft, F. M. (1998) *Diabetes* **47,** 1412–1418
33. Schwanstecher, M., Schwanstecher, C., Dickel, C., Chudziak, F., Moshiri, A., and Panten, U. (1994) *Br. J. Pharmacol.* **113,** 903–911
34. Anzai, N., Izumida, I., Inagaki, N., Seino, S., and Kawahara, K. (1997) *Jpn. J. Physiol.* **47,** S10–S11
35. McNicholas, C. M., Yang, Y., Giebisch, G., and Hebert, S. C. (1996) *Am. J. Physiol.* (*Renal Physiol.*) **271,** F275–F285
36. Leung, Y. M., Zeng, W. Z., Liou, H. H., Solaro, C. R., and Huang, C. L. (2000) *J. Biol. Chem.* **275,** 10182–10189
37. Konstas, A. A., Koch, J. P., Tucker, S. J., and Korbmacher, C. (May 6, 2002) *J. Biol. Chem. 10.1074/jbc.M201925200*