Nicorandil is a new antianginal agent that potentially may be used to treat the cardiovascular side effects of diabetes. It is both a nitric oxide donor and an opener of ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels in muscle and thereby causes vasodilation of the coronary vasculature. The aim of this study was to investigate the domains of the K\(_{\text{ATP}}\) channel involved in nicorandil activity and to determine whether nicorandil interacts with hypoglycemic sulfonylureas that target K\(_{\text{ATP}}\) channels in pancreatic \(\beta\)-cells. K\(_{\text{ATP}}\) channels in muscle and \(\beta\)-cells share a common pore-forming subunit, Kir6.2, but possess alternative sulfonylurea receptors (SURs; SUR1 in \(\beta\)-cells, SUR2A in cardiac muscle, and SUR2B in smooth muscle). We expressed recombinant K\(_{\text{ATP}}\) channels in Xenopus oocytes and measured the effects of drugs and nucleotides by recording macroscopic currents in excised membrane patches. Nicorandil activated Kir6.2/SUR2A and Kir6.2/SUR2B but not Kir6.2/SUR1 currents, consistent with its specificity for cardiac and smooth muscle K\(_{\text{ATP}}\) channels. Drug activity depended on the presence of intracellular nucleotides and was impaired when the Walker A lysine residues were mutated in either nucleotide-binding domain of SUR2. Chimeric studies showed that the COOH-terminal group of transmembrane helices (TM5), especially TM17, is responsible for the specificity of nicorandil for channels containing SUR2. The splice variation between SUR2A and SUR2B altered the off-rate of the nicorandil response. Finally, we showed that nicorandil activity was unaffected by gliclazide, which specifically blocks SUR1-type K\(_{\text{ATP}}\) channels, but was severely impaired by glibenclamide and glimepiride, which target both SUR1 and SUR2-type K\(_{\text{ATP}}\) channels.

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Nicorandil is currently undergoing clinical trials and is being increasingly used for the treatment of angina (1). It has a dual action, operating both as a nitric oxide-generating agent and as an opener of ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels in vascular smooth and cardiac muscle (2). The K\(_{\text{ATP}}\) channels in these tissues are closely related to those that regulate insulin secretion from pancreatic \(\beta\)-cells (3). Like the \(\beta\)-cell channel, they are blocked by many of the sulfonylureas that are widely used to treat type 2 diabetes (4). Because type 2 diabetes increases the risk of cardiovascular disease, many diabetic patients might benefit from therapy with both sulfonylureas and nicorandil. The current study was undertaken to investigate the molecular basis for the previously reported tissue specificity of nicorandil and to determine whether significant interactions might occur between nicorandil and sulfonylureas when both drugs are administered concurrently.

K\(_{\text{ATP}}\) channels respond to alterations in the metabolic activity of the cell and thereby act as sensors of glucose and oxygen availability. In pancreatic \(\beta\)-cells, K\(_{\text{ATP}}\) channels are open at low glucose concentrations; their closure in response to an increase in glucose metabolism leads to membrane depolarization, thereby triggering the entry of calcium and release of insulin (5). The opening of K\(_{\text{ATP}}\) channels in vascular smooth muscle results in muscle relaxation and vasodilation (6). In cardiac muscle, the K\(_{\text{ATP}}\) channels are believed to be closed under normal physiological conditions; their opening during hypoxia results in a protective shortening of the cardiac action potential that reduces cardiac work (7). In all these tissues, the K\(_{\text{ATP}}\) channels are believed to respond to changes in the concentrations of adenine nucleotides.

The molecular composition of K\(_{\text{ATP}}\) channels differs among tissue types and provides a basis for targeting drugs to a specific organ. The pore of the channel is formed from four inwardly rectifying potassium channel subunits (Kir6.2, but Kir6.1 in some smooth muscle channels) (8–10), and ATP inhibits K\(_{\text{ATP}}\) channels by direct interaction with Kir6.2 (11). An additional regulatory subunit, the SUR, endows the channels with sensitivity to Mg nucleotides (which enhance channel activity) and therapeutic drugs such as the sulfonylureas and K\(_{\text{ATP}}\) channel openers (3,12). The SUR, a member of the ATP-binding cassette (ABC) transporter family, is a large membrane protein containing 17 transmembrane helices (TM5s), arranged in three groups of five, six, and six (13). It also possesses two intracellular nucleotide-binding domains (NBDs). Each K\(_{\text{ATP}}\) channel contains four Kir and four

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ABC, ATP-binding cassette; ANOVA, analysis of variance; K\(_{\text{ATP}}\) channel, ATP-sensitive K\(^+\) channel; NBD, nucleotide-binding domain; SUR, sulfonylurea receptor; TM, transmembrane helix.
SUR subunits (3). Two genes encoding SURs have been identified. SUR1 is predominantly expressed in pancreatic β-cells and some neurons (14,15). Alternative splicing of SUR2 produces SUR2A (in cardiac muscle) and SUR2B (in smooth muscle) (16,17). SUR1 and SUR2 share 71% identity; SUR2A and SUR2B differ only in their COOH-terminal 42 amino acids.

In recent years, a number of sites on SURs critical for the binding and regulatory activity of nucleotides, sulfonylureas, and K<sub>ATP</sub> channel openers have been identified. The effect of nucleotides is dependent on their interaction with Walker A and Walker B motifs in the NBDs (18,19). By contrast, the most important domain for drug binding appears to be the COOH-terminal group of six TMs. Binding sites for glibenclamide and the K<sub>ATP</sub> channel opener P1075 (an analog of pinacidil) have been mapped in the cytoplasmic loop connecting TMs 15 and 16 (20). Two additional amino acids in TM 17 (T1253 and L1249 of SUR2) have been implicated in the SUR2-selective action of cromakalim analogs (22). However, homologous residues of SUR1 and SUR2 are also involved in drug binding, as diazoxide and the nonsulfonylurea moiety of glibenclamide exhibit similar potencies on K<sub>ATP</sub> channel subtypes in vitro (4,23). Domains important for the action of nicorandil have not yet been identified.

In this study, we investigated the effects of nicorandil on recombinant K<sub>ATP</sub> channels by expressing the different channel subtypes in Xenopus oocytes. Currents were recorded in inside-out membrane patches in response to the addition of nucleotides and drugs to the intracellular membrane surface. Our results confirmed previous studies that have shown that nicorandil action is dependent on the presence of intracellular nucleotides and further suggested how this might be mediated by the NBDs of SURs. We used SUR chimeras to show that the last TM of SUR2 is critical for nicorandil action, whereas the splice variation between SUR2A and SUR2B modifies the potency of the drug by altering its off-rate. Finally, we showed that sulfonylureas are not homogenous in their ability to interfere with nicorandil activation: although glibenclamide and glimepiride abolished the stimulatory effect of nicorandil, this was not the case for gliclazide. Our data therefore suggest that for patients requiring both nicorandil and sulfonylurea therapy, the most appropriate choice of sulfonylurea might be one that, like gliclazide, preferentially inhibits SUR1-type K<sub>ATP</sub> channels.

**RESULTS**

K<sub>ATP</sub> channels Kir6.2/SUR1 (β-cell type), Kir6.2/SUR2A (cardiac type), and Kir6.2/SUR2B (smooth muscle type) were heterologously expressed in Xenopus oocytes. In all cases, the currents were small in cell-attached membrane patches because of the inhibitory effect of cytoplasmic nucleotides but increased when the patches were excised into nucleotide-free solution.

**Nucleotide dependence of nicorandil activation.** Figure 1 shows that nicorandil had very little effect on Kir6.2/SUR2A or Kir6.2/SUR2B currents in the absence of added nucleotide, as previously reported for native K<sub>ATP</sub> channels (25). In this respect, therefore, the action of nicorandil contrasts with that of pinacidil and its analog P1075, which are effective even in the absence of nucleotide (26,27). Nicorandil (100 μmol/l) activated both types of K<sub>ATP</sub> channel in the presence of ATP, and the relative activation at different ATP concentrations was similar for both SUR2 isofoms (Table 1). However, in the presence of high nucleotide concentrations, which themselves produced strong current inhibition, the absolute increase in current induced by nicorandil was correspondingly smaller. The effect of nicorandil on Kir6.2/SUR2A currents in the presence of 300 μmol/l ATP plus 100 μmol/l ADP was not significantly different from that in 100 μmol/l of ATP alone. The smaller relative activation of Kir6.2/SUR2B currents in 300 μmol/l ATP plus 100 μmol/l ADP may have reflected the fact that the currents reached a maximum level of activation.

**Effects of Walker mutations.** The nucleotide requirement for the action of other K<sub>ATP</sub> channel openers is determined by the NBDs of the SUR subunit (18,19,26–28). Mutation of the Walker A lysine residue in NBD1 or NBD2 of SUR1 impairs nucleotide binding and hydrolysis, as in...
other ABC transporters (29–33). When the Walker A lysine was mutated in NBD1 of SUR2A or SUR2B, pinacidil activation became dependent on the presence of added nucleotide (26,27). However, mutation of the equivalent residue in NBD2 of SUR2A or SUR2B did not impair the amplitude of pinacidil activation.

We therefore investigated the role of the Walker A lysines in the action of nicorandil. Figure 2 shows that mutating the Walker A lysine in NBD1 (K707A) of SUR2A or SUR2B abolished nicorandil activation in the presence of 100 mM ATP. By contrast, mutating the Walker A lysine in NBD2 (K1348A) of SUR2A or SUR2B impaired, but did not completely eliminate, the effect of nicorandil. Nicorandil action requires the last TM of SUR2. The action of nicorandil is specific for K<sub>ATP</sub> channels containing one of the SUR2 isoforms, as Kir6.2/SUR1 currents were unaffected by 100 μmol/l nicorandil in solutions containing ATP alone (300 μmol/l) or ATP plus ADP (300 and 100 μmol/l, respectively) (Fig. 3). This difference between SUR1 and SUR2 enabled us to use a chimeric approach in identifying regions of SUR2 critical for the activity of nicorandil. Chimeric SURs, in which different domains were swapped between SUR1 and SUR2, were coexpressed with Kir6.2 in Xenopus oocytes. We tested the effect of nicorandil in the presence of 300 μmol/l ATP alone or 300 μmol/l ATP plus 100 μmol/l ADP, and expressed the results relative to the conductance in nucleotide-free solution.

Figure 3 shows that nicorandil sensitivity could be introduced into SUR1 by transferring TMs 13–17 from SUR2 to SUR1 (chimera SUR1212). The last TM (TM 17) was crucial for this effect, as nicorandil activity was not transferred by swapping TMs 13–16 alone (chimera SUR129). The reverse chimera, in which TMs 13–16 were swapped from SUR1 to SUR2 (chimera SUR219), was also

### Table 1

<table>
<thead>
<tr>
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<th>Kir6.2/SUR2A</th>
<th>Kir6.2/SUR2B</th>
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<tr>
<td>100 μmol/l ATP</td>
<td>4.5 ± 0.5 (28)*</td>
<td>3.7 ± 0.7 (18)*</td>
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<tr>
<td>300 μmol/l ATP</td>
<td>3.3 ± 0.9 (7)*</td>
<td>5.1 ± 0.6 (17)*</td>
</tr>
<tr>
<td>300 μmol/l ATP + 100 μmol/l ADP</td>
<td>4.4 ± 0.3 (6)*</td>
<td>1.3 ± 0.1 (6)</td>
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Data are means ± SE (n). Current in the presence of nicorandil plus nucleotide is expressed relative to the current in nucleotide alone.

*Not significantly different by ANOVA.
activated by nicorandil, but to a lesser extent than wild-type SUR2A. This was more clearly seen when ADP was added. These data suggest that regions within both TM4, 13–16 and TM17 are essential for the full effect of nicorandil. Similar regions have been reported as essential for the binding of another KATP channel opener, [3H]P1075 (27), and for the activity of cromakalim analogs (22, 34, 35).

**NiCorandil is more effective on SUR2B than SUR2A.**

Previous studies have shown that the reversal rates of pinacidil and P1075 activation are dramatically slowed by nucleotide interaction with the NBDs of SUR, and that this effect is greater for Kir6.2/SUR2B than Kir6.2/SUR2A channels (27). It was postulated that the slower off-rate of P1075 accounts for the higher affinity of the drug for SUR2B reported in binding studies, which are routinely performed in the presence of ATP. The affinity of nicorandil was also greater for SUR2B than SUR2A when measured by the displacement of [3H]P1075 (K<sub>d</sub> ∼8 and ∼19 μmol/l, respectively) (36). Because SUR2A and SUR2B differ only in their final 42 amino acids, a region that does not include the domains that have been implicated in binding studies (27), we investigated whether the different binding affinities of nicorandil might be associated with differences in the off-rate of the drug.

Figure 4A shows that in the presence of 100 μmol/l ATP, the off-rate of nicorandil was considerably slower for Kir6.2/SUR2B currents (time constant, τ ∼25 s) than for Kir6.2/SUR2A currents (τ ∼4 s). This would be consistent with the higher affinity of the drug for SUR2B. The further addition of 100 μmol/l ADP did not significantly alter the nicorandil off-rates (Fig. 4B). Mean data are given in Fig. 4C.

**Effects of different sulfonylureas on nicorandil activation.**

We have previously shown that glibenclamide blocks both Kir6.2/SUR1 and Kir6.2/SUR2A channels with similar potency at a high-affinity site, whereas gliclazide blocks only K<sub>ATP</sub> channels containing the SUR1 subunit with high potency (23, 37). Because many patients require concurrent therapy for angina and diabetes, we next investigated whether the different sulfonylureas interfere with nicorandil activation.

Figure 5 compares the effect of nicorandil in the absence and presence of three different sulfonylureas. The sulfonylureas were tested at concentrations sufficient to saturate the high-affinity site on the SUR subunit. All the solutions contained ATP, and the conductance in the presence of the drug was expressed relative to that in ATP alone. The stimulatory effect of 100 μmol/l nicorandil on Kir6.2/SUR2A and Kir6.2/SUR2B channels was not altered by the presence of a therapeutic concentration of gliclazide (10 μmol/l) but was severely impaired by both glibenclamide (100 nmol/l) and glimepiride (100 nmol/l).

**Discussion**

In this study, we identified regions of the sulfonylurea receptor SUR2 that are responsible for the tissue-specific effects of nicorandil, and showed that the greater potency of nicorandil on Kir6.2/SUR2B (smooth muscle type) compared with Kir6.2/SUR2A (cardiac muscle type) chan-

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**FIG. 3. Effects of chimeras. A:** Cartoon of chimeras. Grey regions indicate SUR1 and black regions indicate SUR2A. **B:** Macroscopic conductance recorded from patches excised from oocytes coexpressing Kir6.2 and SUR1, SUR2A, SUR2B, or indicated SUR chimera, in the presence of 300 μmol/l ATP (○) or 300 μmol/l ATP plus 100 μmol/l nicorandil (■). Mean conductance in the presence of the test solution (G<sub>c</sub>) is expressed relative to the mean conductance in the control (nucleotide and drug-free) solution before and after the addition of agents (G<sub>c</sub>). The dashed line indicates the relative conductance in control solution. The number of patches is given above each bar. *P < 0.05; **P < 0.01; ***P < 0.001; NS, nonsignificant (using a single-sample t test of the percent increase by nicorandil).

**FIG. 4:** Comparison of effects of nicorandil on SUR2B reported in binding studies, which are routinely performed in the presence of ATP. The affinity of nicorandil was also greater for SUR2B than SUR2A when measured by the displacement of [3H]P1075 (K<sub>d</sub> ∼8 and ∼19 μmol/l, respectively) (36). Because SUR2A and SUR2B differ only in their final 42 amino acids, a region that does not include the domains that have been implicated in binding studies (27), we investigated whether the different binding affinities of nicorandil might be associated with differences in the off-rate of the drug.

**FIG. 5:** Comparison of effects of nicorandil on SUR2B reported in binding studies, which are routinely performed in the presence of ATP. The affinity of nicorandil was also greater for SUR2B than SUR2A when measured by the displacement of [3H]P1075 (K<sub>d</sub> ∼8 and ∼19 μmol/l, respectively) (36). Because SUR2A and SUR2B differ only in their final 42 amino acids, a region that does not include the domains that have been implicated in binding studies (27), we investigated whether the different binding affinities of nicorandil might be associated with differences in the off-rate of the drug.
The effects of several sulfonylureas and KATP channel openers are modified by the interaction of Mg nucleotides with the NBDs of SURs (18,19,26–28,38). Nicorandil is no exception, and its stimulatory effect on Kir6.2/SUR2A and Kir6.2/SUR2B channels requires the presence of ATP at the intracellular membrane surface. Previous studies on native KATP channels in cardiac muscle have suggested that nicorandil action is also dependent on the presence of MgADP (25). Our data show that although ADP enhances the absolute current increase induced by the drug, ATP is sufficient to support nicorandil action.

The effects of the Walker A mutations suggest an underlying mechanism to explain the nucleotide dependency of nicorandil action. Previous studies have concluded that ATP remains bound at NBD1 for several minutes after patch excision and that this is prevented by mutation of the Walker A lysine (26,39). In the current study, mutating the Walker A lysine in NB1 abolished nicorandil activation, even in the presence of added ATP, suggesting that nucleotide binding at this NBD is crucial for activity of the drug. Because nicorandil was ineffective in the absence of added nucleotide, even when applied immediately after patch excision, it appears that ATP binding at NBD1 is not sufficient to support nicorandil activation.

Mutation of the Walker A lysine in NBD2 impaired but did not completely abolish nicorandil activation. Other studies have shown that NBD2 of SUR2 is able to hydrolyze ATP and that mutating the Walker A lysine to alanine reduced, but did not completely prevent, nucleotide hydrolysis at NBD2 (33,40). Our results therefore suggest that binding and/or hydrolysis of nucleotide at NBD2, in addition to that at NBD1, is required for nicorandil action.

The nucleotide requirements of nicorandil differ from those of pinacidil, which appears to be effective even when nucleotide is bound only at NBD1 (26). In this respect, nicorandil action more closely resembles that of diazoxide, which, in the case of Kir6.2/SUR2A currents, is also enhanced in the presence of added ADP (41).

**Measuring the nicorandil off-rate.** The splice variation between SUR2A and SUR2B alters only the COOH-terminal 42 amino acids and results in channels that exhibit different responses to nucleotides (17,27,42). The principal effect of the splice variation on the action of nicorandil was to modify the drug off-rate. Previous studies have shown that pinacidil activation of Kir6.2/SUR2B currents reverses more slowly than that of Kir6.2/SUR2A currents, and that this rate is dependent on nucleotide interactions with both NBDs (26,27). The different nicorandil off-rates measured for channels containing SUR2A and SUR2B may therefore be explained by an interaction between nucleotide binding at the NBDs and a drug-binding site in the COOH-terminal group of TMs of SUR2. The slower nicorandil off-rate recorded for Kir6.2/SUR2B compared with Kir6.2/SUR2A currents is reflected in the greater affinity of SUR2B for nicorandil measured in binding studies (36).

The affinities of SUR2A and SUR2B for nicorandil, measured in binding studies by the displacement of [3H]P1075, were 19 and 8 μmol/l, respectively (36). Similar values have been reported for native cardiac and smooth muscle tissues (36). Although the potency of nicorandil on Kir6.2/SUR2B channels expressed in HEK293 cells was also in this range (EC_{50} = 10 μmol/l), this was not the case for Kir6.2/SUR2A channels (EC_{50} > 500 μmol/l) (43). Our results are more compatible with those obtained in binding studies, as we did not observe any further increase in the response of Kir6.2/SUR2A currents to nicorandil when the drug concentration was increased from 100 μmol/l to 1 mmol/l (data not shown). We also found that the splice variation between SUR2A and SUR2B altered the nicorandil off-rate approximately fivefold, consistent with the moderate difference in the affinity between SUR2A and SUR2B found in binding studies. A possible explanation for the greater EC_{50} of nicorandil measured for Kir6.2/
SUR2A currents in HEK293 cells is the presence of the high (3 mmol/l) intracellular ATP concentration used in these experiments (43), as our data showed that nicorandil activation is modulated by the nucleotide concentration. The intracellular solution in whole cell experiments may also contain other factors that modulate channel activity.

**Identification of domains critical for nicorandil activity.** The specificity of nicorandil for K<sub>ATP</sub> channels containing SUR2 isoforms enabled us to use a chimeric approach to identifying regions of SURs important for activity of the drug. We showed that the final TM (TM 17) of SUR2 is essential for nicorandil activity but that the preceding four TMs are also required to confer a full drug response. The chimeric approach has been previously used to show that the COOH-terminal group of TMs is also involved in the binding of radiolabeled glibenclamide to SUR1 and of P1075 to SUR2 (20,21). The final TM helix was shown to be crucial for both [3H]P1075 binding and cromakalim activity (21,22,35), although it was not required for pinacidil activity (12).

**Interaction between nicorandil and sulfonylureas.** Our results clearly showed that nicorandil action on Kir6.2/SUR2A and Kir6.2/SUR2B channels is severely impaired by glibenclamide and glimepiride but is unaffected by gliclazide. The lack of interference by gliclazide is not surprising, as this drug blocks β-cell but not cardiac or smooth muscle types of K<sub>ATP</sub> channel (37). In contrast, glibenclamide and glimepiride block all three types of K<sub>ATP</sub> channels with high affinity (23,44). Although glibenclamide and glimepiride can reverse the stimulatory effect of nicorandil, we cannot distinguish whether this is because the drugs impair nicorandil binding or interfere with the transduction of nicorandil binding into channel opening. The former idea, however, is supported by the fact that glibenclamide displaces the binding of [3H]P1075 from SUR2 (36).

**Conclusion.** The differential potency of nicorandil between tissues can be explained by sequence variations in TMs 13–17 and the last 42 amino acids of the SUR. The effect of nicorandil is impaired by concomitant administration of glibenclamide or glimepiride, but is not affected by gliclazide. The results thus clearly indicate that care should be taken in clinical practice when prescribing combinations of K<sub>ATP</sub> channel openers and sulfonylureas, because drugs like glibenclamide may impair the clinical effectiveness of nicorandil.

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**FIG. 5. Interference of sulfonylurea and nicorandil action.** Macroscopic currents were recorded from oocytes coexpressing Kir6.2 and either SUR2A or SUR2B. Nicorandil (100 μmol/l) was applied in the presence of ATP (100 μmol/l), but 300 μmol/l in the case of Kir6.2/SUR2B currents with glimepiride. After activation reached steady state, gliclazide (10 μmol/l), glibenclamide (100 nmol/l), or glimepiride (100 nmol/l) was also added. The mean conductance in the presence of nicorandil or nicorandil plus sulfonylurea (G) is expressed relative to the mean conductance measured in the presence of ATP alone (G<sub>ATP</sub>). The conductance in the presence of ATP (and ATP plus nicorandil) is given as the average of conductances measured before and after application of the sulfonylurea. The number of patches is given above each bar. *P < 0.01; **P < 0.001; NS, nonsignificant (using a paired t test to compare the conductance in ATP plus nicorandil and ATP plus nicorandil plus sulfonylurea).


