

The Novel Diazoxide Analog 3-Isopropylamino-7-Methoxy-4*H*-1,2,4-Benzothiadiazine 1,1-Dioxide Is a Selective Kir6.2/SUR1 Channel Opener

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ATP-sensitive K⁺ (K_{ATP}) channels are activated by a diverse group of compounds known as potassium channel openers (PCOs). Here, we report functional studies of the Kir6.2/SUR1 Selective PCO 3-isopropylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (NNC 55-9216). We recorded cloned K_{ATP} channel currents from inside-out patches excised from *Xenopus laevis* oocytes heterologously expressing Kir6.2/SUR1, Kir6.2/SUR2A, or Kir6.2/SUR2B, corresponding to the β-cell, cardiac, and smooth muscle types of the K_{ATP} channel. NNC 55-9216 reversibly activated Kir6.2/SUR1 currents (EC₅₀ = 16 μmol/l). This activation was dependent on intracellular MgATP and was abolished by mutation of a single residue in the Walker A motifs of either nucleotide-binding domain of SUR1. The drug had no effect on Kir6.2/SUR2A or Kir6.2/SUR2B currents. We therefore used chimeras of SUR1 and SUR2A to identify regions of SUR1 involved in the response to NNC 55-9216. Activation was completely abolished and significantly reduced by swapping transmembrane domains 8–11. The reverse chimera consisting of SUR2A with transmembrane domains 8–11 and NBD2 consisting SUR1 was activated by NNC 55-9216, indicating that these SUR1 regions are important for drug activation. [³H]glibenclamide binding to membranes from HEK293 cells transfected with SUR1 was displaced by NNC 55-9216 (IC₅₀ = 105 μmol/l), and this effect was impaired when NBD2 of SUR1 was replaced by that of SUR2A. These results suggest NNC 55-9216 is a SUR1-selective PCO that requires structural determinants, which differ from those needed for activation of the K_{ATP} channel by pinacidil and cromakalim. The high selectivity of NNC 55-9216 may prove to be useful for studies of the

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ATP-sensitive K⁺ (K_{ATP}) channels are found in a variety of tissues, including endocrine, muscle, and neural tissues, where they couple the metabolic state of the cell to its electrical activity. In pancreatic β-cells, K_{ATP} channels link changes in blood glucose concentration to insulin secretion (1); in cardiac myocytes, increased K_{ATP} channel activity during ischemia may protect against myocardial damage by shortening the action potential (2); in vascular smooth muscle, K_{ATP} channels regulate vessel tone (3); and in skeletal muscle, they contribute to the enhanced K⁺ efflux and fatigue found during severe exercise (4). Their physiological role in neurons is not clearly established, but it is believed that they modulate electrical activity (5), influence synaptic transmitter release (6), and contribute to the response to cerebral ischemia (7).

The K_{ATP} channel is an octameric complex of two different protein subunits, an inwardly rectifying K-channel, Kir6.x, and a sulfonylurea receptor, SURx (8–14). The former acts as the K_{ATP} channel pore, whereas SUR is a channel regulator that endows Kir6.x with sensitivity to drugs and Mg nucleotides (15). K_{ATP} channels in different tissues are composed of different subunits (16). In most cases, the pore comprises Kir6.2 (8,10,17), but in some smooth muscles, it is Kir6.1 (18). These Kir subunits associate with different types of SUR. The β-cell K_{ATP} channel is composed of SUR1 and Kir6.2 (8,10), the cardiac type of SUR2A and Kir6.2 (11), and the smooth muscle type of SUR2B combined with either Kir6.1 or Kir6.2 (17,18). Both Kir6.2/SUR1 and Kir6.2/SUR2B combinations are found in the brain (5,19). Although wild-type K_{ATP} channels require both SUR and Kir6.x for functional activity, a mutant form of Kir6.2 with a COOH-terminal truncation of 26 or 36 amino acids (Kir6.2ΔC) is capable of independent expression (15,20). Kir6.2ΔC therefore provides a useful tool for studying the effects of drugs on the pore-forming subunit of the K_{ATP} channel.

K_{ATP} channels are inhibited by sulfonylureas and activated by a chemically diverse group of compounds known as potassium channel openers (PCOs) (21–23). These effects are mediated by interaction of the drug with the SUR subunit of the channel. It is well established that different types of K_{ATP} channels exhibit markedly different

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ABC, ATP-binding cassette; K_{ATP}, ATP-sensitive K⁺; NBD, nucleotide-binding domain; NNC 55-9216, 3-isopropylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide; PCO, potassium channel opener.

sensitivities toward the various PCO drugs. In particular, pinacidil and cromakalim activate both native and cloned cardiac (Kir6.2/SUR2A) and smooth muscle (Kir6.2/SUR2B) types of K_{ATP} channel, but have little or no effect on β -cell (Kir6.2/SUR1) channels (3,24–26). Conversely, diazoxide strongly activates β -cell and smooth muscle K_{ATP} channels and has only a weak stimulatory effect on cardiac K_{ATP} channels (27–30).

Nucleotides modulate K_{ATP} channel activity by interaction with both SUR and Kir6.2 subunits. Binding of ATP or ADP to an unidentified site on Kir6.2 causes channel closure (15,31). In contrast, Mg nucleotides enhance K_{ATP} channel activity and decrease its sensitivity to ATP by interaction with the nucleotide-binding domains (NBDs) of the SUR subunit (32–35). Interaction of nucleotides with the NBDs of SUR also modifies the response to PCOs. For example, stimulation of β -cell K_{ATP} channels by diazoxide requires the presence of intracellular Mg nucleotide (36), and mutations in the NBDs of SUR1 abolish the ability of MgATP to support diazoxide activation (33,35). Likewise, although pinacidil can activate cardiac K_{ATP} channels in the absence of added nucleotide (provided that the drug is tested before channel run-down is pronounced) (21,37,38), both the rate of deactivation of Kir6.2/SUR2A currents on removal of pinacidil and the ability to measure binding of the analog [3 H]P-1075 to SUR2A require the presence of MgATP and intact NBDs (38–40). One explanation for the MgATP dependence of PCO activation of K_{ATP} channels is offered by the observation that PCOs enhance the ATPase activity of NBD2 of SUR2 (41). It is proposed that this promotes the duration of the nucleotide-bound state at one or both NBDs and thereby stimulates K_{ATP} channel activation.

A β -cell-specific PCO would be useful for treatment of inoperable insulinoma and some forms of persistent hypoglycemic hyperinsulinemia of infancy. Furthermore, it has been proposed that β -cell “rest” induced by PCOs might be beneficial in the treatment of type 1 diabetes (42–44). β -Cell-specific PCOs have also been suggested for prevention of type 2 diabetes and obesity (45–47). By combining structural elements from pinacidil and diazoxide and by reducing the conformational flexibility compared with pinacidil, a range of novel diazoxide analogs have been synthesized and shown to inhibit insulin release from pancreatic islets (48–51). In screening tests, the compound 3-isopropylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (NNC 55-9216 or BPDZ 216) (Fig. 1) was a more potent inhibitor of insulin release from rat pancreatic islets than diazoxide and had no significant myorelaxant action on rat aortic rings in vitro (data on file at Novo Nordisk and the Free University of Brussels). These results suggested NNC 55-9216 might show specificity for the β -cell K_{ATP} channel. In this study, we investigated the effect of NNC 55-9216 on recombinant K_{ATP} channels composed of Kir6.2 and either SUR1 (β -cell type), SUR2A (cardiac/skeletal muscle type), or SUR2B (smooth muscle type), heterologously expressed in *Xenopus* oocytes. A chimeric approach was used to investigate the domains of SUR that are involved in NNC 55-9216-induced K_{ATP} channel activation. This identified a novel PCO interaction site in the second set of transmembrane domains of SUR1.

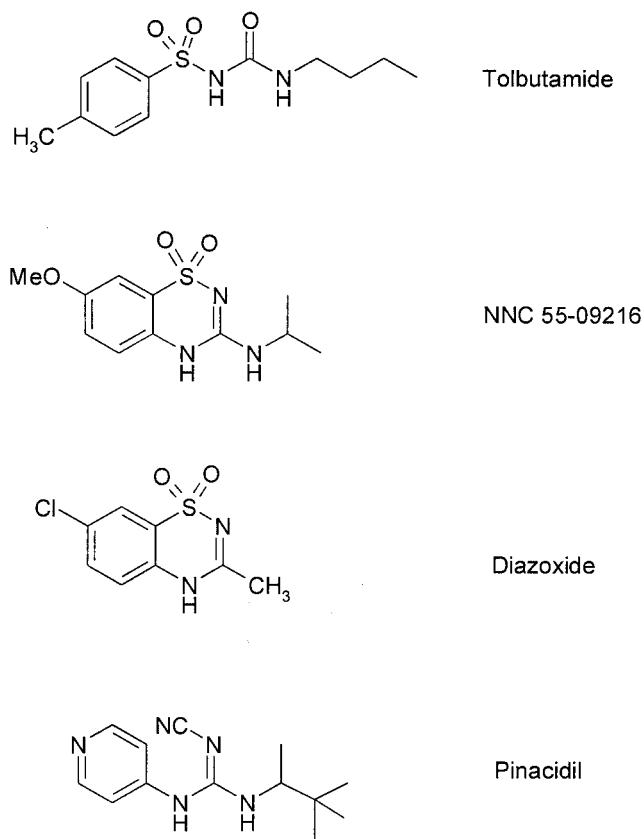


FIG. 1. Structures of tolbutamide, NNC 55-9216, diazoxide, and pinacidil.

RESEARCH DESIGN AND METHODS

Molecular biology. Mouse Kir6.2 (Genbank no. D50581) (8,10), rat SUR1 (Genbank no. L40624) (9), rat SUR2A (Genbank no. D83598) (11), and SUR2B (Genbank no. D86038) (17) cDNAs were cloned in the pBF vector. A truncated form of Kir6.2 (Kir6.2 Δ C36), which lacks the COOH-terminal 36 amino acids and forms functional channels in the absence of SUR, was prepared as previously described (15). SUR chimeras containing different segments of SUR1 and SUR2A were constructed using standard molecular biology techniques (52). The chimeras were composed of the following segments, in which the numbers refer to sequences of SUR1 or SUR2A, as appropriate. Chimeras based on SUR1 are denoted SUR12-*a* to SUR12-*e*, and chimeras based on SUR2A are denoted SUR21-*a* to SUR21-*f*: SUR12-*a*: (1-662;SUR1)-(655-849;SUR2A)-(871-1581;SUR1); SUR12-*b*: (1-1284;SUR1)-(1249-1545;SUR2A); SUR12-*c*: (1-662;SUR1)-(655-849;SUR2A)-(871-1284;SUR1)-(1249-1545;SUR2A); SUR12-*d*: (1-423;SUR1)-(417-651;SUR2A)-(660-1581;SUR1); SUR12-*e*: (1-1034;SUR1)-(1013-1241;SUR2A)-(1278-1581;SUR1); SUR21-*a*: (1-1248;SUR2A)-(1285-1581;SUR1); SUR21-*b*: (1-648;SUR2A)-(656-870;SUR1)-(850-1248;SUR2A)-(1285-1581;SUR1). SUR21-*c*: (1-414;SUR1)-(408-1545;SUR2A); SUR21-*d*: (1-416;SUR2A)-(424-700;SUR1)-(689-1545;SUR2A); SUR21-*e*: (1-1012;SUR2A)-(1035-1277;SUR1)-(1242-1545;SUR2A); and SUR21-*f*: (1-416;SUR2A)-(424-700;SUR1)-(689-1248;SUR2A)-(1285-1581;SUR1).

Mutagenesis of individual amino acids was performed using the altered sites II System (Promega). Capped mRNA was prepared using the mMES-SAGE mMACHINE large-scale in vitro transcription kit (Ambion, Austin, TX), as previously described (53). When chimeric SURs were coexpressed with Kir6.2 in *Xenopus* oocytes, current amplitudes were similar to those found for wild-type Kir6.2/SUR1 or Kir6.2/SUR2A channels.

Oocyte collection. Female *Xenopus laevis* were anesthetized with MS222 (2 g/l added to the water). One ovary was removed via a mini-laparotomy, the incision was sutured, and the animal was allowed to recover. After the wound had completely healed, the second ovary was removed in a similar operation, and the animal was then killed by decapitation while under anesthesia. Immature stage-V-VI oocytes were incubated for 60 min with 1.0 mg/ml collagenase (type V; Sigma) and manually defolliculated. Oocytes were either injected with \sim 1 ng Kir6.2 Δ C36 mRNA or coinjected with \sim 0.1 ng Kir6.2 mRNA and \sim 2 ng mRNA encoding wild-type or chimeric SUR. The final injection volume was 50 nl/oocyte. Isolated oocytes were maintained in Barth's solution and studied 1–5 days after injection (53).

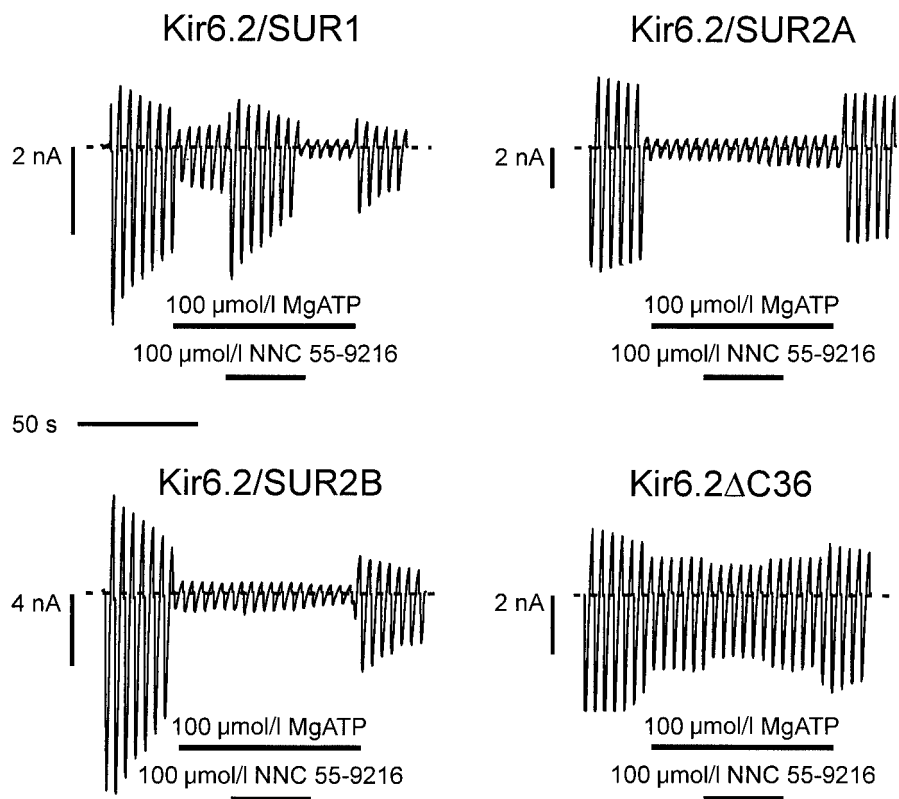


FIG. 2. Effects of NNC 55-9216 on Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B, or Kir6.2ΔC36 currents. Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to 100 mV from oocytes coexpressing Kir6.2 and either SUR1, SUR2A, or SUR2B or expressing Kir6.2ΔC36. MgATP and NNC 55-9216 were added to the intracellular solution as indicated by the bars.

Electrophysiology. Patch pipettes were pulled from thick-walled borosilicate glass and had resistances of 250 – 500 k Ω when filled with pipette solution. Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV and at 20 – 24°C (53). Currents were evoked by repetitive 3 -s voltage ramps from -110 mV to 100 mV and recorded using an EPC7 patch-clamp amplifier (List Elektronik, Darmstadt, Germany). They were filtered at 0.2 kHz, digitized at 0.4 kHz using a Digidata 1200 Interface, and analyzed using pClamp software (Axon Instruments, Foster City, CA).

The pipette (external) solution contained (mmol/l): 140 KCl, 1.2 MgCl₂, 2.6 CaCl₂, and 10 HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained (mmol/l): 110 KCl, 1.4 MgCl₂, 10 EGTA, and 10 HEPES (pH 7.2 with KOH; final $[\text{K}^+]$ ~ 140 mmol/l). NNC 55-9216 (synthesized at the Department of Medicinal Chemistry, University of Liege) was prepared as a 200 mmol/l stock solution in DMSO. In control experiments, the maximal DMSO concentration applied (0.15%) was without effect on the K_{ATP} channel current. Solutions containing ATP, ADP, or ATP γ S were made fresh each day. The pH of the solution was readjusted after addition of the drug and/or nucleotide. 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.

Preparation of SUR-expressing membranes. HEK293 cells were grown to 60 – 70% confluency in 13 -cm petri dishes and transfected using FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Confluent dishes of transfected HEK293 cells were washed once in PBS, harvested with a cell scraper, and centrifuged at $1,000g$ in PBS for 5 min. All subsequent procedures were performed at $\sim 0^{\circ}\text{C}$. The supernatant was removed, and cells were resuspended in ice-cold buffer (buffer 1: 30 mmol/l HEPES and 2 mmol/l MgCl₂, pH 7.4 with NaOH), homogenized with an Ultra Turrax T25 homogenizer, and centrifuged at $48,000g$ for 15 min. The pellet was resuspended, and the homogenization step was repeated. The final pellet was resuspended in buffer 1 (500 – $1,500$ μl per dish) with 250 mmol/l sucrose. The membrane solution was aliquoted and stored at -80°C for later use in binding assays.

[^3H]glibenclamide binding. Membranes were thawed, placed on ice, and diluted in ice-cold buffer (30 mmol/l HEPES, 2 mmol/l MgCl₂, and 2 mmol/l Na₂ATP; pH 7.4 adjusted with NaOH) to a concentration of 200 – 800 $\mu\text{g}/\text{ml}$ protein. The radioligand [^3H]glibenclamide (52.00 Ci/mmol) (NEN, Boston, MA) was added to a final concentration of 1 nmol/l. [^3H]glibenclamide was displaced with increasing concentrations of NNC 55-9216 (1 – $1,000$ $\mu\text{mol}/\text{l}$). Samples were incubated at 37°C for 1 h. Incubation was terminated by

filtration through Whatman GF/B filters, and the filters were washed three times with 154 mmol/l of ice-cold NaCl. Ultima Gold was used as the scintillation fluid for counting ^3H decay. Nonspecific binding was determined using 10 $\mu\text{mol}/\text{l}$ glipizide (final concentration). The ratio of nonspecific binding to total [^3H]glibenclamide bound was always <0.15 , and the amount of total [^3H]glibenclamide, bound as a function of protein concentration, was linear over the protein concentration range used (200 – 800 $\mu\text{g}/\text{ml}$).

Data analysis. The slope conductance was measured by fitting a straight line to the current-voltage relation between -20 and -100 mV. Conductance was measured from an average of five consecutive ramps in each solution. Responses to NNC 55-9216 or nucleotides were expressed relative to the conductance measured in control solution without added NNC 55-9216 or nucleotides. Concentration-response curves were constructed by expressing the conductance in the presence of NNC 55-9216 (G) as a fraction of that in control solution (G_c). The following equation was then fitted to the data (54):

$$\frac{G}{G_c} = L + \frac{1 - L}{1 + ([\text{NNC 55-9216}]/\text{EC}_{50})^h}$$

where G and G_c are the slope conductances in the presence of NNC 55-9216 and in control solution, respectively, $[\text{NNC 55-9216}]$ is the concentration of NNC 55-9216, EC_{50} is the NNC 55-9216 concentration at which activation is half maximal, h is the Hill coefficient (slope factor), and L is the maximum activation. Data were fit using Microcal Origin software.

To measure the time course of the NNC 55-9216 response, patches were held at -60 mV and macroscopic currents were recorded in response to application of the drug.

In the binding experiments, a modified form of the Hill equation was used to fit the data:

$$y = L + \frac{1 - L}{1 + ([\text{NNC 55-9216}]/\text{IC}_{50})^h}$$

where y is the number of [^3H]glibenclamide counts at a given NNC 55-9216 concentration expressed as a percentage of that in the absence of NNC 55-9216 both corrected for nonspecific binding, $[\text{NNC 55-9216}]$ is the NNC 55-9216 concentration, IC_{50} is the NNC 55-9216 concentration at which half the [^3H]glibenclamide is displaced, h is the Hill coefficient (slope factor), and L is the nondisplaceable fraction of [^3H]glibenclamide.

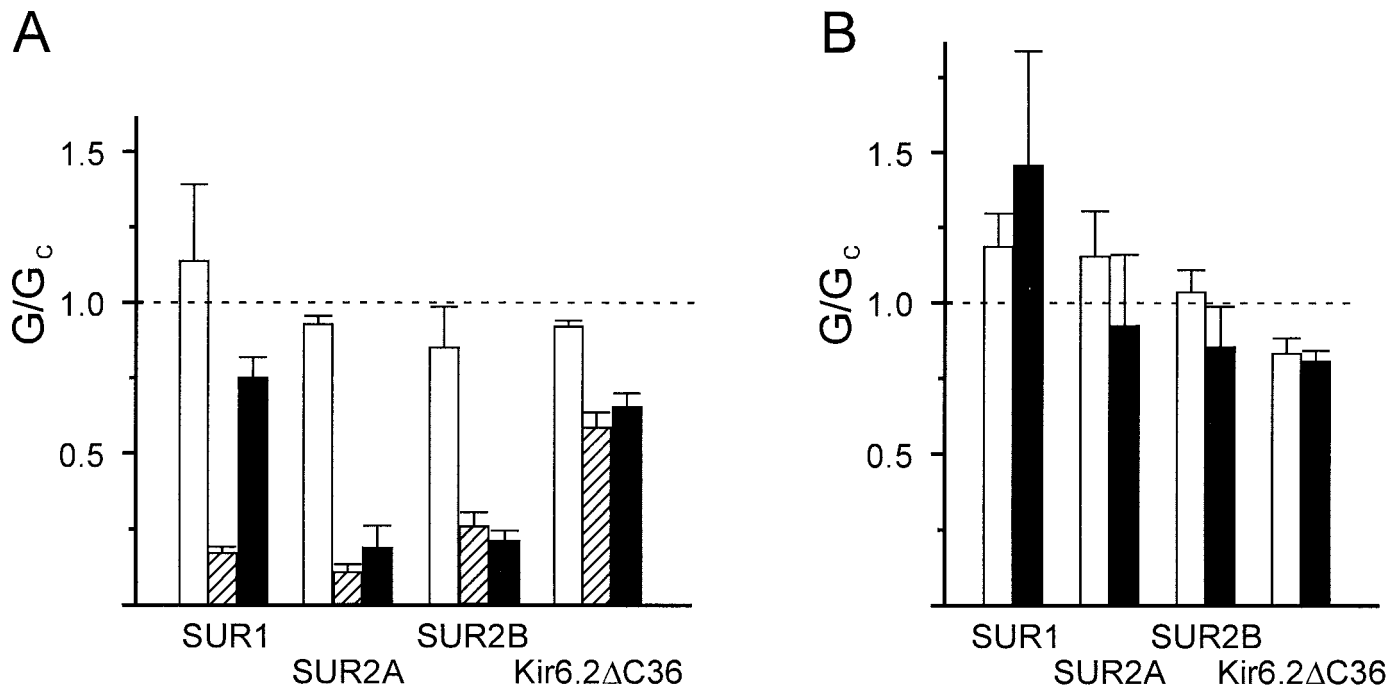


FIG. 3. Nucleotide dependence of NNC 55-9216 action. **A:** Mean macroscopic slope conductances (G) in the presence of 100 $\mu\text{mol/l}$ NNC 55-9216 and no nucleotide (\square), 100 $\mu\text{mol/l}$ MgATP (\square), or 100 $\mu\text{mol/l}$ MgATP + 100 $\mu\text{mol/l}$ NNC 55-9216 (\blacksquare). **B:** Mean macroscopic slope conductances (G) in the presence of 10 $\mu\text{mol/l}$ MgADP (\square) or 10 $\mu\text{mol/l}$ MgADP + 100 $\mu\text{mol/l}$ NNC 55-9216 (\blacksquare). Data are expressed as a fraction of the mean slope conductance in control solution (no additions) (G_c) ($n = 3\text{--}33$). The vertical bars indicate 1 SEM. The dashed line indicates the conductance in control solution (no additions).

All data are presented as means \pm 1 SEM. Statistical significance was tested using Student's t test.

RESULTS

We examined the ability of NNC 55-9216 to activate the different types of recombinant K_{ATP} channels by recording macroscopic currents in inside-out membrane patches excised from *Xenopus* oocytes coexpressing Kir6.2 and either SUR1, SUR2A, or SUR2B or expressing Kir6.2 Δ C36 alone. In all cases, the currents were very small in the cell-attached configuration but increased dramatically when the patch was excised into nucleotide-free solution, consistent with the idea that in the intact oocyte, the K_{ATP} channel is blocked by cytoplasmic nucleotides such as ATP. Because PCOs usually require MgATP to support their action, we tested NNC 55-9216 in the presence of 100 $\mu\text{mol/l}$ MgATP. This ATP concentration blocked Kir6.2 Δ C36 currents by \sim 40% and Kir6.2/SUR1 currents by \sim 83% (Figs. 2 and 3).

Figure 2 shows that when 100 $\mu\text{mol/l}$ NNC 55-9216 was added to the intracellular membrane surface, it largely reversed the block of Kir6.2/SUR1 currents produced by 100 $\mu\text{mol/l}$ MgATP but had no effect on Kir6.2/SUR2A, Kir6.2/SUR2B, or Kir6.2 Δ C36 currents. The concentration-response relation for NNC 55-9216 activation of Kir6.2/SUR1 currents measured in the presence of 100 $\mu\text{mol/l}$ MgATP is shown in Fig. 4. The data were fit by the Hill equation (equation 1) with an EC_{50} of 16 ± 8 $\mu\text{mol/l}$ and a Hill coefficient of 0.8 ± 0.2 ($n = 6\text{--}16$). The fact that the Hill coefficient is close to unity suggests that the binding of a single molecule of the drug is sufficient to cause channel activation. In all subsequent experiments, NNC 55-9216 was used at a concentration of 100 $\mu\text{mol/l}$ because this appeared to produce a near-maximal response.

Interactions with Mg-nucleotides. We explored whether MgATP is required to support activation of Kir6.2/SUR1 currents by NNC 55-9216 by testing the efficacy of the drug in the absence of added nucleotide (Fig. 3, white columns). Similar experiments were carried out using Kir6.2/SUR2A, Kir6.2/SUR2B, and Kir6.2 Δ C36. As Fig. 3 shows, the drug failed to activate all four types of K_{ATP} channel in the absence of MgATP.

The nucleotide diphosphate MgADP is able to support diazoxide activation of Kir6.2/SUR1 (33). Furthermore, although diazoxide is relatively ineffective at activating Kir6.2/SUR2A in the presence of MgATP, the drug produces reliable activation of Kir6.2/SUR2A channels in the presence of MgADP (28). We therefore tested the effects of NNC 55-9216 on all four types of K_{ATP} channel in the presence of 10 $\mu\text{mol/l}$ MgADP. This nucleotide concentration was chosen because higher concentrations produce near-maximal activation of the channel, which may mask any stimulatory effect of the drug itself. Figure 3 shows that NNC 55-9216 was unable to activate Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B, or Kir6.2 Δ C36 channels when MgADP was present. NNC 55-9216 (100 $\mu\text{mol/l}$) was also tested under conditions where diazoxide activates Kir6.2/SUR2A channels [i.e., in the presence of 100 $\mu\text{mol/l}$ MgADP plus 100 $\mu\text{mol/l}$ MgATP (28)] but no stimulatory effect was observed on currents through Kir6.2/SUR2A channels ($n = 3$, data not shown). These data indicate that the presence of intracellular MgATP, rather than MgADP, is required for channel activation by NNC 55-9216 and provide additional support that the drug is selective for the β -cell type of K_{ATP} channel.

We next investigated the mechanism by which MgATP

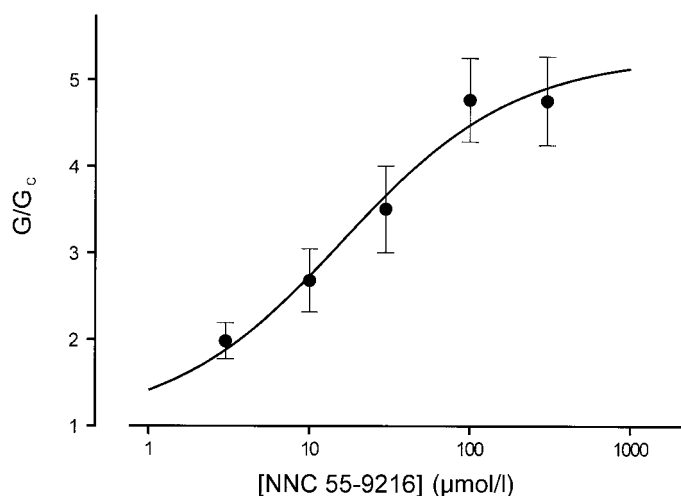


FIG. 4. NNC 55-9216 concentration-response relation for Kir6.2/SUR1 currents ($n = 6-16$). Mean macroscopic slope conductance in the presence of NNC 55-9216 plus 100 $\mu\text{mol/l}$ MgATP (G), expressed as a fraction of the mean slope conductance in control solution containing 100 $\mu\text{mol/l}$ MgATP but not NNC 55-9216 (G_c). The vertical bars indicate 1 SEM. The solid line is the best fit of the Hill equation (equation 1) to the data: $EC_{50} = 16 \pm 8 \mu\text{mol/l}$, $h = 0.8 \pm 0.2$ ($n = 6-16$).

supports activation of Kir6.2/SUR1 channels by NNC 55-9216. Fig. 5A compares the effect of 100 $\mu\text{mol/l}$ NNC 55-9216 in the presence of 100 $\mu\text{mol/l}$ MgATP, in the presence of 100 $\mu\text{mol/l}$ ATP but without Mg^{2+} , and in the presence of 100 $\mu\text{mol/l}$ MgATP γ S. As has been observed for diazoxide (36,55), Mg^{2+} was required for the ability of ATP to support channel activation by NNC 55-9216. Although this result is consistent with the idea that ATP hydrolysis is needed for NNC 55-9216 activation, we cannot exclude the alternative possibility that MgATP, but not ATP alone, is able to interact with the channel. Figure 5A also shows that ATP γ S was able to substitute partially for ATP, albeit with lower efficacy (40% activation compared with 75%). There was no difference in the rate of activation by 100 $\mu\text{mol/l}$ NNC 55-9216 in the presence of ATP γ S or ATP, but the rate of deactivation of Kir6.2/SUR1 currents on removal of the drug was significantly slower in ATP solution (Fig. 5B). The mean time constants were $12.6 \pm 0.7 \text{ s}$ ($n = 8$) and $6.4 \pm 0.8 \text{ s}$ ($n = 5$) for solutions containing 100 $\mu\text{mol/l}$ MgATP or MgATP γ S, respectively. This indicates that the off-rate of the drug is slower when ATP is present, a finding that may reflect a slower rate of hydrolysis of ATP γ S compared with ATP, a reduced binding affinity of ATP γ S, or a difference in the extent of coupling between binding/hydrolysis and channel activation.

To further explore the role of MgATP in channel activation by NNC 55-9216, we made mutations in the NBDs of SUR1. Specifically, we mutated lysine 719 to alanine [SUR1(K719A)] and lysine 1384 to methionine [SUR1(K1384M)]. These residues lie in the Walker A motifs of NBD1 and NBD2, respectively, and, in other ATP-binding cassette (ABC) transporters, their mutation impairs ATP binding and/or hydrolysis. Coexpression of either of these mutant SUR1s with Kir6.2 produced currents of similar amplitude to wild-type currents (Fig. 6). As previously reported, the currents were slightly more ATP sensitive (33). They were also not activated by NNC

55-9216, even in the presence of MgATP. These results imply that the ability of MgATP to support channel activation by NNC 55-9216 results from interaction of the nucleotide with the NBDs of SUR1. Furthermore, both NBDs must be intact for channel activation. In this respect, NNC 55-9216 differs from diazoxide, which is still able to induce some activation of Kir6.2/SUR1(K1384M) currents (33).

Identification of the NNC 55-9216 binding site. As described above, NNC 55-9216 activates Kir6.2/SUR1 but not Kir6.2/SUR2A channels (Figs. 2 and 3). We used this difference to identify the region of SUR1 that confers sensitivity to the drug by constructing chimeric SURs based on either SUR1 or SUR2A (Fig. 7A). These chimeric SURs were coexpressed with Kir6.2, and the ability of 100 $\mu\text{mol/l}$ NNC 55-9216 to activate the expressed currents in the presence of 100 $\mu\text{mol/l}$ MgATP was then examined (Fig. 7B).

Transfer of NBD1 of SUR2 into SUR1 (chimera 12-a) had little effect, whereas substitution of NBD2 from SUR2 to SUR1 (12-b) markedly reduced NNC 55-9216 stimulation. Moreover, when both NBD1 and -2 of SUR1 were replaced with those of SUR2 (12-c), the effect of the drug was completely abolished. These findings suggest that NBD2 of SUR1 is essential for NNC 55-9216 activation. We next investigated the effects of replacing various transmembrane helices of SUR1 with the equivalent region of SUR2A. Figure 7B also shows that although transfer of TMs 13-16 from SUR2A to SUR1 (12-e) confers pinacidil sensitivity on SUR1 (37,56,57), it did not affect the stimulatory effect of NNC 55-9216. In contrast, the efficacy of NNC 55-9216 was approximately halved when TMs 8-11 of SUR1 were replaced with the corresponding TMs of SUR2A (12-d). This suggests that TMs 8-11 are important for NNC 55-9216 sensitivity.

We also constructed chimeric SURs based on SUR2A, in which the equivalent region of SUR1 replaced various domains in SUR2A. Swapping TMs 1-7 (21-c), 8-11 (21-d), or 13-16 (21-e) did not confer NNC 55-9216 sensitivity on SUR2A. Likewise, transfer of either NBD2, or both NBDs, of SUR1 into SUR2A did not render NNC 55-9216 capable of activating the K_{ATP} channel current. However, when both TMs 8-11 and NBD2 were transferred from SUR1 into SUR2A (21-f), a significant activation by NNC 55-9216 was seen ($36 \pm 7\%$, $n = 9$), thus confirming the importance of these domains for NNC 55-9216 activation.

Because NNC 55-9216 shares some structural similarities with the inhibitory sulfonylurea tolbutamide (Fig. 1), we examined whether a single amino acid mutation that abolishes tolbutamide sensitivity (SUR1-S1238Y) (52) was able to abolish activation by NNC 55-9216. Wild-type and mutant K_{ATP} channels, preblocked by 100 $\mu\text{mol/l}$ MgATP, were activated by NNC 55-9216 (100 $\mu\text{mol/l}$) to similar extents: to $75 \pm 7\%$ ($n = 33$) and $61 \pm 15\%$ ($n = 5$) of control (nucleotide-free solution), respectively. This result indicates that the drug interaction sites for tolbutamide and NNC 55-9216 are not identical, a view also supported by the fact that TMs 13-16 of SUR1, which form part of the tolbutamide-binding site (52), do not appear to be involved in NNC 55-9216 activation.

Displacement of [^3H]glibenclamide by NNC 55-9216. We next explored the ability of NNC 55-9216 to displace [^3H]glibenclamide binding to membranes isolated from

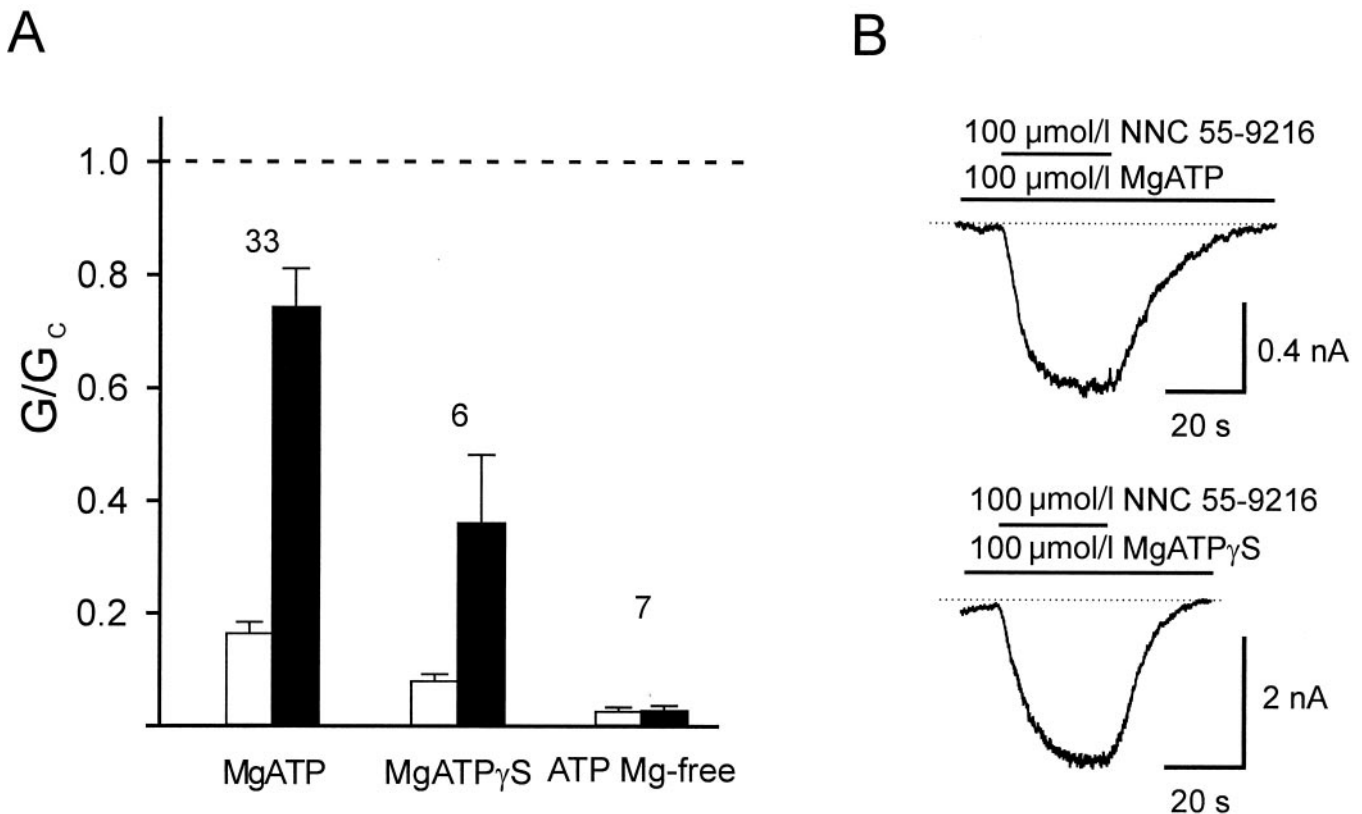


FIG. 5. Effects of nucleotide hydrolysis on NNC 55-9216 activation of Kir6.2/SUR1 currents. **A:** Mean macroscopic slope conductance in the presence of 100 μ mol/l MgATP, 100 μ mol/l MgATP γ S, or 100 μ mol/l Mg-free ATP in the absence (\square) or presence (\blacksquare) of 100 μ mol/l NNC 55-9216. Conductance (G) is expressed as a fraction of the mean slope conductance in control solution without added drug or nucleotide (G_c). The numbers above the columns indicate the number of patches and the vertical bars indicate 1 SEM. The dashed line indicates the control level (100%). **B:** Effect of NNC 55-9216 on Kir6.2/SUR1 currents recorded at -60 mV in the presence of 100 μ mol/l MgATP and 100 μ mol/l MgATP γ S. Drug and nucleotide were added to the intracellular surface as indicated by the bars.

HEK293 cells transiently transfected with SUR1, or with the chimeras 12-*a* or 12-*b* (consisting of SUR1 with either NBD1 or NBD2 of SUR2A, respectively). All experiments were carried out in the presence of 100 μ mol/l MgATP, because PCO binding to SUR requires the presence of MgATP (58). Figure 8 shows that NNC 55-9216 dose-dependently displaced [3 H]glibenclamide binding to SUR1, with an IC_{50} of 103 ± 11 μ mol/l ($n = 6$). A similar result was obtained for displacement of [3 H]glibenclamide binding to the chimera 12-*a* by NNC 55-9216 (Fig. 8). However, NNC 55-9216 was less effective at displacing [3 H]glibenclamide from chimera 12-*b*. Thus, swapping NBD1 of SUR1 for that of SUR2A is without obvious effect, whereas replacement of NBD2 of SUR1 by that of SUR2A has a marked effect. This is consistent with the results of the electrophysiological experiments.

It was not possible to dissolve NNC 55-9216 at concentrations >1 mmol/l; hence, the full concentration-response relationship for the 12-*b* chimera could not be obtained. It is not clear from the available data whether the displacement curve corresponds to a single curve or a multimodal curve, or whether incomplete displacement of binding occurs at saturating concentrations of the PCO. If the shifted curve is a result of binding of the PCO at a single site, the data can be a fit with an IC_{50} of 730 ± 25 μ mol/l and $h = 0.43 \pm 0.07$ ($n = 5$). Alternatively, the data are also consistent with the idea that NNC 55-9216 is only able to displace a maximum of 50% of [3 H]glibenclamide binding.

In this case, the IC_{50} was 51 ± 30 μ mol/l and $h = 0.76 \pm 0.21$ ($n = 5$). Whichever is correct, it is clear that replacement of NBD2 of SUR1 by that of SUR2A reduces the ability of NNC 55-9216 to displace [3 H]glibenclamide binding.

DISCUSSION

The results reported here demonstrate that NNC 55-9216 activates Kir6.2/SUR1, but not Kir6.2/SUR2A or Kir6.2/SUR2B currents, by interaction with the SUR1 subunit of the K_{ATP} channel. They also imply that the drug will stimulate native K_{ATP} channels in pancreatic β -cells but not in cardiac or smooth muscles. This selectivity is particularly interesting in view of the fact that the drug shares some structural similarity with diazoxide, which interacts with K_{ATP} channels containing SUR2A and SUR2B as well as SUR1 (28,59).

Activation of Kir6.2/SUR1 currents by NNC 55-9216 is dependent on the presence of intracellular MgATP. The adenine dinucleotide MgADP is unable to substitute for MgATP, and in this respect, the nucleotide requirements of NNC 55-9216 differ from those of diazoxide (Fig. 1), which is supported by MgADP (28). The lack of effect of MgADP, and of ATP in the absence of Mg^{2+} , suggests that MgATP hydrolysis might be needed for NNC 55-9216 activation. The ability of MgATP γ S to support the action of the diazoxide analog, albeit to a lesser extent than MgATP, is also consistent with a role for ATP hydrolysis. In this case,

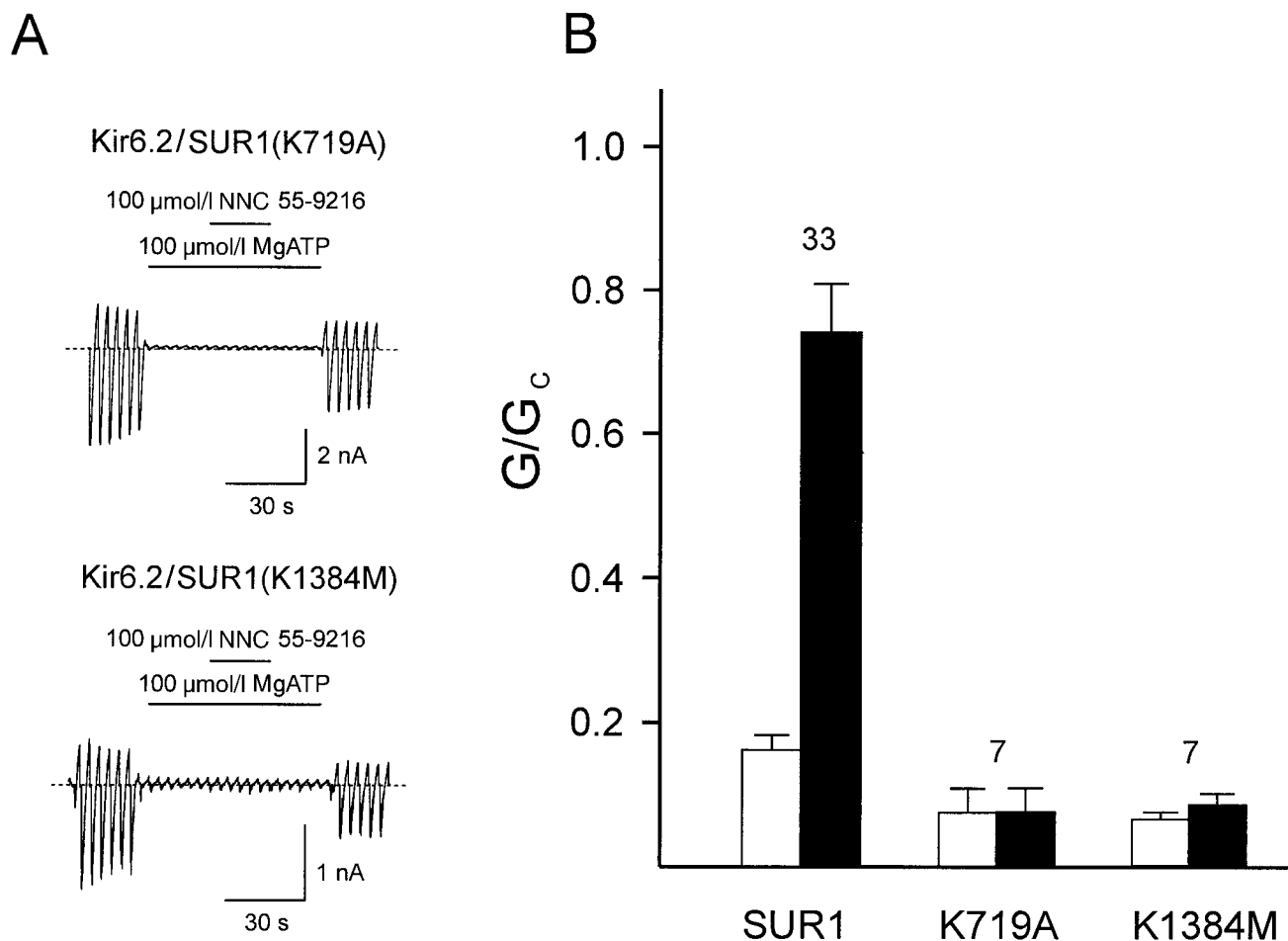


FIG. 6. Effects of Walker A mutations on NNC 55-9216 activity. **A:** Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to 100 mV from oocytes coexpressing Kir6.2 and either SUR1(K719A) or SUR1(K1384 mol/l). MgATP and NNC 55-9216 were added to the intracellular solution as indicated by the bars. **B:** Mean macroscopic slope conductances (G) in the presence of 100 μmol/l MgATP (□) or 100 μmol/l MgATP + 100 μmol/l NNC 55-9216 (■), expressed as a fraction of the mean slope conductance in control solution without drug or nucleotide (G_c). The numbers above the columns indicate the number of patches, and the vertical bars indicate 1 SEM.

the faster off-rate of the drug in ATP γ S solution might also be explained by a slower hydrolysis of MgATP γ S (compared with MgATP). However, we cannot exclude the possibility that MgATP binding per se (rather than MgATP binding with subsequent hydrolysis) is sufficient to support NNC 55-9216 stimulation, and that the slower off-rate of the drug in the presence of MgATP results because the allosteric effect on drug binding differs for MgATP γ S and MgATP.

Mutagenesis experiments suggest that MgATP supports NNC 55-9216 activation by interaction with the NBDs of SUR1. Both NBDs are required because mutation of a single lysine residue in the Walker A motif of either NBD1 or NBD2 was sufficient to abolish the effect of the drug. In other ABC transporters, mutation of the Walker A lysine prevents ATP hydrolysis without markedly altering ATP binding (60,61). Similar mutations in SUR1, however, decrease ATP binding (62,63). Our mutagenesis experiments, therefore, cannot distinguish whether ATP binding or hydrolysis is required for NNC 55-9216 activation.

The experiments using SUR1/2A chimeras highlight the importance of several domains of SUR1 for channel activation by NNC 55-9216. First, it is clear that NBD2 plays a particularly important role because substitution of this

domain by the equivalent region of SUR2A abolished the effect of NNC 55-9216. This is in sharp contrast to diazoxide, which activates equally well when NBD2 of SUR1 is replaced by that of SUR2A (27). It is possible that the NBDs of SUR1 cooperate in nucleotide binding (and/or transduction), that this interaction is needed for nucleotides to support NNC 55-9216 activation, and that NBD2 of SUR2A is unable to interact with that of NBD1. Second, replacement of TMs 8–11 of SUR1 with the equivalent region of SUR2A abolished the action of the drug. There are three principal mechanisms by which manipulation of TMs 8–11 might influence the stimulatory effect of NNC 55-9216. First, it may disrupt the drug binding site; second, it may influence the coupling mechanism by which drug binding is transduced into channel opening; and third, it may impair the ability of MgATP to support NNC 55-9216 binding and/or activation. The available data do not allow us to distinguish between these possibilities. We were able to transfer some of the activity of NNC 55-9216 from SUR1 to SUR2A by substituting both TMs 8–11 and NBD2 from SUR1 into SUR2A. The efficacy of NNC 55-9216 activation is reduced in this chimera, but the presence of some activation confirms that TMs 8–11 of SUR1 are required for proper NNC 55-9216 function.

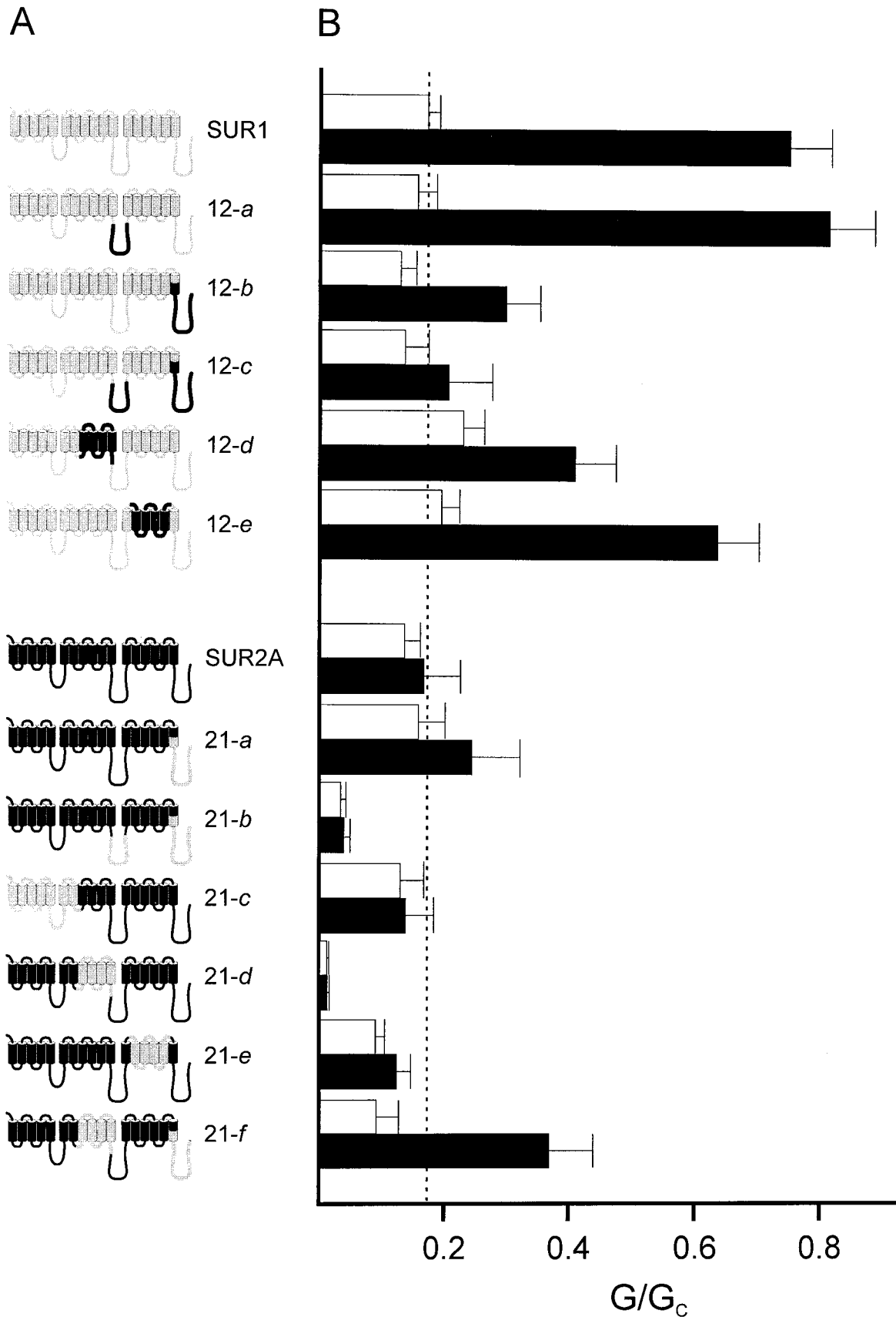


FIG. 7. Effects of NNC 55-9216 on K_{ATP} channels containing chimeric SURs. **A:** Schematic representation of SUR chimeras indicating the regions exchanged between SUR1 and SUR2A. **B:** Mean macroscopic slope conductance (G) in the presence of 100 $\mu\text{mol/l}$ MgATP (\square) or 100 $\mu\text{mol/l}$ MgATP + 100 $\mu\text{mol/l}$ NNC 55-9216 (\blacksquare), expressed as a fraction of the mean slope conductance in control solution (no added drug or nucleotide) (G_C) ($n = 6-33$). The vertical bars indicate 1 SEM. The dashed line shows the mean Kir6.2/SUR1 current in 100 $\mu\text{mol/l}$ MgATP for comparative purposes.

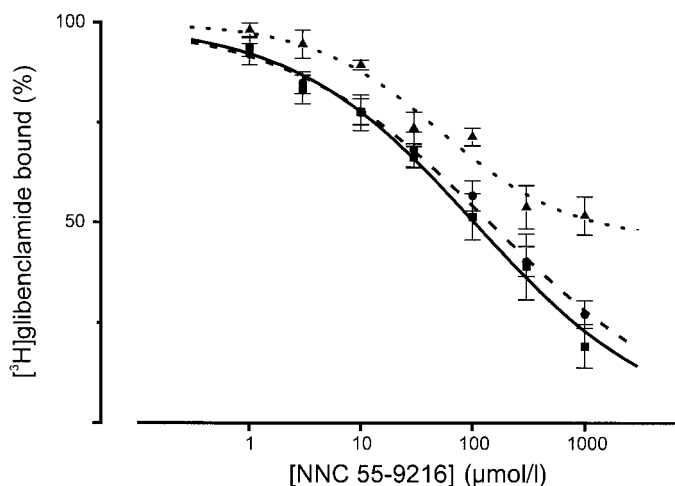


FIG. 8. Displacement of [3 H]glibenclamide by NNC 55-9216 from HEK 293 membranes expressing SUR1, chimera 12- α , or chimera 12- β . Each data point is the mean of 5–6 duplicate determinations that were corrected for nonspecific binding and normalized to the maximal specific binding. The curves are fitted with the Hill equation (equation 2) using the following values: SUR1: $L = 0$, $IC_{50} = 103 \mu\text{mol/l}$, and $h = 0.53$ ($n = 6$) (■, solid line); chimera 12- α : $L = 0$, $IC_{50} = 142 \mu\text{mol/l}$, and $h = 0.48$ ($n = 6$) (●, dashed line); and chimera 12- β : $L = 0.46$, $IC_{50} = 51 \mu\text{mol/l}$, and $h = 0.76$ ($n = 5$) (▲, dotted line.).

The SUR subunit consists of 17 TMs arranged in three groups of 5, 6, and 6 (64,65). Previous studies have demonstrated that a range of PCOs that show specificity for SUR2, including pinacidil, cromakalim, and nicorandil, interact with the third set of TMs. Chimera studies suggest that pinacidil and P1075 interact with TMs 13–16 of SUR2A (38,56,57). Mutation of two residues within TM 17 [SUR2A (L1249T) and SUR2A(T1253M)] abolishes activation of Kir6.2/SUR2A currents by the cromakalim analog SR47063, whereas the reverse mutation SUR1(M1290T) confers sensitivity to P1075, rilmakalim, and SR47063 on Kir6.2/SUR1(M1290T) (25). These mutations did not abolish activation by diazoxide, however, suggesting that it may interact with a different site. Our studies now identify the presence of a novel interaction site for PCOs that lies in the second set of transmembranes of SUR1 (specifically TMs 8–11). It is possible that in addition to NNC 55-9216, diazoxide may interact with (part of) this site. It is interesting that this region lies in the COOH-terminal part of the second set of transmembranes because in the related ABC transporter multidrug resistance protein, drug binding sites are located in an equivalent position in both the second and third set of TMs.

The ability of NNC 55-9216 to displace [3 H]glibenclamide binding to SUR1 in the presence of MgATP suggests several possibilities. First, the binding sites for these drugs may overlap, or they may be able to influence each other allosterically. Second, it is possible that the PCO affects [3 H]glibenclamide binding indirectly, for example, by stabilizing ATP binding to SUR1, which in turn reduces [3 H]glibenclamide binding. The importance of NBD2 for this effect is demonstrated by the reduced displacement that is observed when NBD2 of SUR2A replaces that of SUR1, a finding that supports the results of the electrophysiological experiments. It seems most likely that interaction of MgATP with NBD2 is essential for NNC 55-9216 binding, but we cannot exclude the possibility that

it is instead involved in allosteric coupling of PCO and sulfonylurea binding sites.

In conclusion, NNC 55-9216 is the first PCO that shows significant selectivity for Kir6.2/SUR1 channels (the β -cell type of K_{ATP} channels). It is also more potent than diazoxide ($EC_{50} = 65 \mu\text{mol/l}$ for diazoxide [10] versus $16 \mu\text{mol/l}$ for NNC 55-9216). Of particular interest, is the finding that NNC 55-9216 appears to interact with a different region of SUR than many other PCOs (pinacidil, cromakalim, etc.). This suggests that it may form the basis for the development of Kir6.2/SUR1-selective PCOs that are devoid of the side effects (e.g., edema and hirsutism) associated with diazoxide. Such compounds could be used to treat patients suffering from diseases of excessive insulin secretion, such as insulinoma or certain types of persistent hypoglycemic hyperinsulinemia of infancy. They may also be of value in the treatment of both type 1 (42–44) and type 2 diabetes (45) as well as obesity (46). Finally, NNC 55-9216 may provide a useful pharmacological tool for studies on the molecular mechanisms of PCO action and for studies of the relationship between the structure and function of sulfonylurea receptors.

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