Arylcyanoguanidines as Activators of Kir6.2/SUR1K_{ATP} Channels and Inhibitors of Insulin Release

Tina M. Tagmose,[†] Søren C. Schou,[†] John P. Mogensen,[†] Flemming E. Nielsen,[†] Per O. G. Arkhammar,[†] Philip Wahl,[†] Birgit S. Hansen,[†] Anne Worsaae,[†] Harrie C. M. Boonen,[†] Marie-Hélène Antoine,[‡] Philippe Lebrun,[‡] and John Bondo Hansen*[,†]

Discovery, Novo Nordisk A/S, Novo Nordisk Park, DK 2760, Måløv, Denmark, and Laboratoire de Pharmacodynamie et de Thérapeutique, Université Libre de Bruxelles, 808, Route de Lennik, B-1070 Bruxelles, Belgium

Received September 3, 2003

Phenylcyanoguanidines substituted with lipophilic electron-withdrawing functional groups, e.g. *N*-cyano-*N*′-[3,5-bis-(trifluoromethyl)phenyl]-*N*″-(cyclopentyl)guanidine (**10**) and *N*-cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(3-methylbutyl)guanidine (**12**) were synthesized and investigated for their ability to inhibit insulin release from beta cells, to repolarize beta cell membrane potential, and to relax precontracted rat aorta rings. Structural modifications gave compounds, which selectively inhibit insulin release from βTC6 cells (e.g. compound **10**: $IC_{50} = 5.45 \pm 1.9$ μM) and which repolarize βTC3 beta cells (**10**: $IC_{50} = 4.7 \pm 0.5$ μM) without relaxation of precontracted aorta rings (**10**: $IC_{50} > 300$ μM). Inhibition of insulin release from rat islets was observed in the same concentration level as for βTC6 cells (**10**: $IC_{50} = 1.24 \pm 0.1$ μM, **12**: $IC_{50} = 3.8 \pm 0.4$ μM). Compound **10** (10 μM) inhibits calcium outflow and insulin release from perifused rat pancreatic islets. The mechanisms of action of **10** and **12** were further investigated. The compounds depolarize mitochondrial membrane from smooth muscle cells and beta cell and stimulate glucose utilization and mitochondrial respiration in isolated liver cells. Furthermore, **10** was studied in a patch clamp experiment and was found to activate Kir6.2/SUR1 and inhibit Kir6.2/SUR2B type of K_{ATP} channels. These studies indicate that the observed effects of the compounds on beta cells result from activation of K_{ATP} channels of the cell membrane in combination with a depolarization of mitochondrial membranes. It also highlights that small structural changes can dramatically shift the efficacy of the cyanoguanidine type of selective activators of Kir6.2/SUR2 potassium channels.

Introduction

The ATP-sensitive potassium (K_{ATP}) channels are present in various types of cells such as cardiac, smooth muscle, and pancreatic beta cells. The open-state probability of the K_{ATP} channels, which is regulated by intracellular nucleotides such as ATP and ADP, couples the metabolic state to cellular membrane potential. Pharmacological modulation of the K_{ATP} channels by blockers (e.g. sulfonylurea and benzoic acid insulin secretagogues) and potassium channel openers has provided important drugs for treatment of, for example, metabolic and cardiovascular diseases.[1,2]

The K_{ATP} channel exists as an octameric complex of the sulfonylurea receptor (SUR) and the pore-forming inwardly rectifying potassium channel (Kir) in a 4+4 stoichiometry.[3] The genes for two closely related sulfonylurea receptors SUR1 and SUR2 have been cloned. Two different splice variants of SUR2, SUR2A and SUR2B, have been reported. SUR1 combines with Kir6.2 to form the K_{ATP} channels of pancreatic beta cells, whereas the cardiac type consists of SUR2A and Kir6.2 and the smooth muscle type of SUR2B and Kir6.1 or Kir6.2. Activation of K_{ATP} channels of vascular smooth muscle causes relaxation and subsequently reduction in blood pressure. In pancreatic beta cells high glucose concentrations increase the ATP/ADP ratio to close K_{ATP} channels. This will depolarize the cellular membrane and open the voltage-gated Ca^{2+} (L-type) channels to stimulate insulin secretion. Pharmacological activation of the K_{ATP} channels in the presence of high glucose concentrations will prevent insulin secretion. The weak and nonselective K_{ATP} channel opener (K_{ATP}CO) diazoxide and the SUR1 selective K_{ATP}COs NNC55-0118 and NN414 (Figure 1) inhibit insulin release.[4] The cyanoguanidine pinacidil is a potent and SUR2 selective K_{ATP}CO, which inhibits insulin release only at high concentrations.[4,5] The potassium channel blockers and potassium channel openers interact with the sulfonylurea receptor in a compound specific and complex manner, which involves both transmembrane regions and the two nucleotide binding folds.[1,6,7]

The existence of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels has been proposed.[8] Opening of mitoK_{ATP} results in an increased K^+ influx into the mitochondria, which could result in uncoupling of the respiratory chain due to futile cycling of K^+ via the K^+/H^+ antiporter.[9] In consequence, this will increase energy expenditure and decrease cellular ATP levels, which subsequently will reduce insulin secretion. The molecular structure of mitoK_{ATP} channels is probably multimeric and similar to the plasma membrane K_{ATP} channels.[10] Diazoxide has been shown to exhibit a pronounced differential pharmacology between sar-

* To whom correspondence should be addressed. Telephone: +454-4434857. Fax: +4544434547. E-mail: jbha@novonordisk.com.
[†] Novo Nordisk A/S.
[‡] Université Libre de Bruxelles.

10.1021/jm031018y CCC: $27.50
Published on Web 05/04/2004

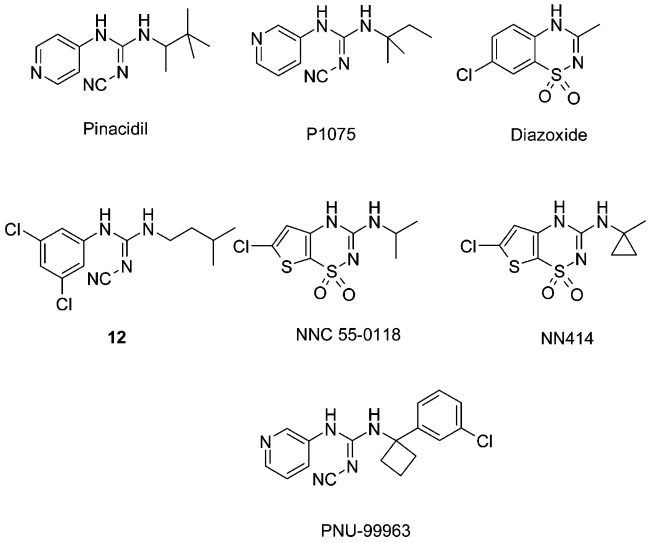


Figure 1. Structures of modulators of ATP-sensitive potassium channels.

colemmal K_{ATP} and mitoK_{ATP} of bovine heart, being about 1000 times more potent at opening mitoK_{ATP} channels.[10] The mitochondrial K_{ATP} channels could play a role in protecting cardiomyocytes during ischemia and reperfusion, and it has been shown by Garlid et al.[11] that diazoxide significantly protected hearts against ischemic injury at concentrations lower than those required for the opening of sarcolemmal K_{ATP}. The protective effect of potassium channel openers only requires modest activation of mitoK_{ATP}, which does not produce any detectable mitochondrial uncoupling.[12]

It has furthermore been found that the cyanoguanidine derivative P1075 uncouples oxidative phosphorylation through activation of mitoK_{ATP}[13] and that high concentrations of pinacidil can uncouple mitochondria by virtue of an intrinsic protonophoretic action.[14]

K_{ATP}COs such as diazoxide, NNC 55-0118, and NN414 are able to preserve beta cell function in vivo[15−17] and in vitro,[18] an effect possibly mediated through an inhibition of insulin release (beta cell rest). In high concentrations, and probably through deactivation of mitochondria, NNC 55-0118 furthermore protects beta cells against streptozotocin, alloxan, and interleukin 1β-induced toxicity.[19,20] Compounds, which are able to inhibit insulin release and depolarize mitochondrial membranes, therefore could have beneficial effects in preservation of beta cell function in patients suffering from type 1 or type 2 diabetes.

We recently found that replacement of the pyrido group in pinacidil with a 3,5-disubstituted phenyl group resulted in compounds which activate beta cell K_{ATP} channels to inhibit glucose-induced insulin secretion at concentrations which do not relax smooth muscle.[21] In this study, we wish to present an exploration of the synthesis and mechanism of action of a series of 3,5-disubstituted phenylcyanoguanidines (Scheme 1).

Chemistry

The most commonly used methods for the preparation of cyanoguanidines are (1) treatment of an aromatic isothiocyanate with an amine to give the corresponding thiourea, conversion of the thiourea to its carbodiimide and treatment of this with cyanamide to give the cyanoguanidine;[22,23] (2) treatment of an aromatic isothiocyanate with cyanamide and conversion of the obtained *N*-cyanothiourea to the cyanoguanidine by treatment with an amine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride;[24] (3) treatment of diphenyl *N*-cyanocarbonimidate[25] or dimethyl *N*-cyanoamido-S,S-dimethylthio-carbamate[26] with an aromatic amine and subsequently treating the obtained *N*-cyano-isourea or *N*-cyano-isothiourea with alkylamines.

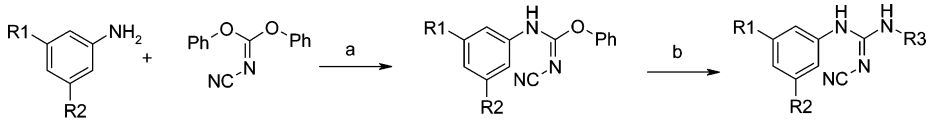
In the synthesis of the present compounds, we used diphenyl *N*-cyanocarbonimidate as starting material as described previously[21] (Scheme 1). The phenylcyanoguanidines were obtained in moderate to good yields.

Results and Discussion

In Vitro Screening. Pinacidil relaxes rat aortic rings precontracted with phenylephrine while it does not inhibit glucose-induced insulin release from βTC6 beta cells nor hyperpolarize βTC3 beta cells, as reported previously.[4] Diazoxide is, in contrast, a weak but nonselective K_{ATP}CO as indicated by the effects on beta cells and vascular tissue.[4] The screening assay for measuring changes in membrane potential of the βTC3 cells has been modified compared to the one that was used previously.[21,27] Using the new procedure, diazoxide was found to be more potent on βTC3 cells compared to what was reported previously.

The size and bulkiness of the *N*-alkyl side chain of the examined 3,5-bistrifluoromethyl cyanoguanidines **2**−**10** is important for the activity of the compounds. With two exceptions, the compounds were able to hyperpolarize the βTC3 cell membrane and to inhibit glucose-induced insulin secretion from βTC6 cells. The *tert*-amylamino (**8**) and the isopropylamino (**4**) derivatives were poorly active on beta cells indicating that two methyl groups α to the nitrogen considerably reduce beta cell activity. The 4-methylbutylamino derivative

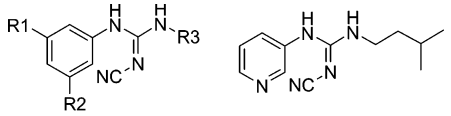
Scheme 1. Synthesis of Phenylcyanoguanidines[a]



1a:R1,R2= CF_3, CF_3
1b:R1,R2= CF_3, OCH_3
1c:R1,R2= Cl, Cl
1d:R1,R2= F, F

[a] Reagents and conditions: (a) RT, 14 h; (b) $R3NH_2$, 75 °C, 8 h.

Table 1. Biological Activity of Compounds **2**−**19** and Reference K_{ATP}COs on Smooth Muscle and Beta Cells



2-18 **19**

			relaxation of rat aorta rings	inhibition of insulin release βTC6 cells		membrane potential βTC3 cells
compound	R1, R2	R3	IC_{50}, μM[a]	IC_{50}, μM[a]	efficacy[a] %	IC_{50}, μM[b]
2	CF_3, CF_3	$CH_2CH_2CH_3$	12.4 ± 2.69	5.0 ± 2.9	53 ± 5.2	19 ± 4
3	CF_3, CF_3	$CH_2CH_2CH(CH_3)_2$	5.6 ± 2.52	2.0 ± 0.1	77 ± 3.6	4.7 ± 0.3
4	CF_3, CF_3	$CH(CH_3)_2$	61 ± 51.8	41.4 ± 11.2	20 ± 9.8	NS
5	CF_3, CF_3	$CH(CH_3)CH(CH_3)_2$	17.6 ± 3.38	5.2 ± 2.3	69 ± 6.7	5.5 ± 0.4
6	CF_3, CF_3	*S*-$CH(CH_3)C(CH_3)_3$	11.9 ± 0.60	13.7 ± 4.6	67 ± 7.8	12 ± 5
7	CF_3, CF_3	*R*-$CH(CH_3)C(CH_3)_3$	20.3 ± 3.19	4.4 ± 0.8	68 ± 5.6	18 ± 6
8	CF_3, CF_3	$C(CH_3)_2CH_2CH_3$	0.9 ± 0.24	NA	-	NS
9	CF_3, CF_3	1-(3-chlorophenyl)cyclobut-1-yl	27.9 ± 6.21	2.8 ± 0.8	66 ± 2.5	20 ± 7.0
10	CF_3, CF_3	cyclopentyl	>300	5.45 ± 1.9	65 ± 5	4.7 ± 0.5
11	OCH_3,CF_3	cyclopentyl	75 ± 30.1	NA	-	NS
12	Cl,Cl	$CH_2CH_2CH(CH_3)_2$	18.7 ± 5.12	4.2 ± 2.0	60 ± 7.9	0.8 ± 0.2
13	Cl,Cl	$CH(CH_3)_2$	52 ± 27.3	NA	-	NS
14	Cl,Cl	*S*-$CH(CH_3)$Ph	30.0 ± 12.8	39.8 ± 13.9	27 ± 4.9	25 ± 10
15	Cl,Cl	*R*-$CH(CH_3)$Ph	43.8 ± 30.1	16.1 ± 5.6	22 ± 7.1	NS
16	Cl,Cl	$C(CH_3)_2CH_2CH_3$	0.3 ± 0.08	48.5 ± 13.2	32 ± 5	24 ± 0.6
17	Cl,Cl	cyclopentyl	75 ± 16.0	28.7 ± 19.4	18 ± 1.2	12 ± 0.4
18	F,F	$CH_2CH_2CH(CH_3)_2$	85 ± 11.7	NA	-	NS
19			11 ± 2.40	1.2 ± 0.8	23 ± 5.9	NS
diazoxide			12.8 ± 2.5[c]	22.4 ± 3.7[c]	23 ± 3.8[c]	13.7 ± 0.25
pinacidil[c]			0.8 ± 0.2	>100		NS

[a] Values are means of at least four experiments ± SEM. [b] Values are means of at least three experiments ± SD. [c] The values are taken from Nielsen et al.[4] (NA = not active, efficacy was found to be less than 10%; NS = not significant).

(**3**) is the most potent compound, being approximately 10 times as potent as diazoxide with respect to both inhibition of insulin release and hyperpolarization of βTC3 cell membranes. There appears to be only small differences between the potencies of the two 1,2,2-trimethylpropylamino (pinacidil-like) enantiomers **6** and **7**. It has previously been shown that modification of the *N*-alkyl side-chain of the pyrido-cyanoguanidine, P1075, reverses the efficacy and produces very potent K_{ATP} channel blockers (e.g. PNU-99963) of vascular smooth muscle.[27] The 1-(3-chlorophenyl)cyclobutylamino side chain taken from PNU 99963[28] in combination with 3,5-bis(trifluoromethyl)phenyl resulted in the potent inhibitor of insulin secretion **9**. In our hands, PNU-99963 did not affect glucose-stimulated insulin release from βTC6 cells or membrane potential of βTC3 cells (data not shown) and had only minimal effects on isolated rat aorta
(EC_{50} = 113 ± 25 μM). Substituting one trifluoromethyl group of **10** with a methoxy group to get **11** considerably reduced beta cell activity.

Changing the trifluoromethyl groups to chloro groups gave compounds **12**−**17** with similar or slightly less potent effects on beta cells. In contrast, the 3,5-difluorophenyl derivative **18** was not active on beta cells, indicating that electron-withdrawing substituents on the phenyl is necessary in order to obtain activity on beta cells. Retaining the 4-methylbutylamino side-chain but substituting the 3,5-bis-(trifluoromethyl)phenyl with a 3-pyrido group (**3** vs **19**) resulted in a considerable loss of efficacy with respect to inhibition of insulin release from βTC6 cells and total loss of effects on βTC3 membrane potential.

The effects of the compounds on rat aortic rings are dependent on the size of the *N*-alkyl substituent and less on the substitutions on the phenyl group. The most potent compounds, which are as potent as pinacidil, have 3,5-dichloro or 3,5-bistrifluoromethyl phenyl groups and the *tert*-amylamino (P1075-like) side chain, **8** and **16**. Furthermore, these two compounds are relatively selective for vascular smooth muscle. The cycloalkyl derivatives **10**, **11**, and **17** had little or no activity on the aortic rings, which make compound **10** beta cell selective.

Table 2. Effects of **10**, **12** on Insulin Release from Rat Islets, Membrane Potential of Mitochondria from Muscle and Beta Cells and Glucose Utilization in Hepatocytes (HEP-G2 cells)

compd	insulin release rat islets IC_{50} (μM) [eff: %][a]	A10 (mito) ED_{50} (μM)[b]	βTC3 (mito) ED_{50} (μM)[c]	HEP-G2 EC_{50} (μM) [E_{max}: % of full stimulation][d]
10	1.24 ± 0.1 [65 ± 8]	1.0 ± 0.4	>30	10.4 ± 0.2 [95 ± 10]
12	3.8 ± 0.4 [72 ± 7.4]	2 ± 5	>30	22 ± 6 [98 ± 16]

[a] Values are an average of two experiments ± SEM. [b] Values are from one experiment with eight data points run in duplicate, the EC_{50} and SD values extracted using WinCurveFit 1.0.2 with a four-parameter nonlogistic curve fit. [c] Values are an average of three experiments. [d] Values are an average of three experiments ± SEM.

The activities of selected compounds on smooth muscle and beta cells have been examined in further detail. **10** and **12** have been tested with respect to inhibition of glucose-stimulated insulin release from isolated rat islets, on membrane potential of mitochondria of A10 smooth muscle cells and βTC3 beta cells and on stimulation of glucose utilization of rat hepatocytes (Table 2).

The compounds reduced glucose-stimulated insulin release from isolated islets. The potency on islets was comparable or higher than on the βTC6 beta cells. In

comparison, on rat islets the IC_{50} for diazoxide averages 20.28 ± 11.7 μM.[4]

The JC-1 fluorescence probe was used to measure changes in membrane potential of mitochondria in the A10 smooth muscle cell line and in the βTC3 cell line. FCCP was used as a positive control. While concentrations of more than 30 μM of either compound were needed in order to significantly affect βTC3 mitochondrial membrane, **10** and **12** potently depolarized the mitochondrial membranes of A10 cells as indicated by the recorded changes in the JC-1 fluorescence (Table 2). The measured changes were in the concentration ranges similar to the ones at which plasma membrane K_{ATP} channel activation (Table 1) and inhibition of insulin release is observed. The discrepancies between the effects of the two compounds on mitochondria from βTC3 versus A10 cells could in part be due to different sensitivities of the experimental methods. In comparison, **10** was found to depolarize mitochondrial membranes of isolated rat or mouse islets at concentrations above 10 μM (data not shown, P. O. G. Arkhammar, unpublished; S. Sandler, unpublished). Furthermore, compounds **10** and **12** were tested for stimulation of state-4 respiration in liver mitochondria. The compounds increase O_2 respiration in a concentration range from 1 to 10 μM. The effect observed in the liver mitochondria can be due to several effects; an activation of mitochondria K_{ATP} channels, interference with mitochondria transport systems including exchange of a proton, a protonophoric uncoupling, inhibition of the respiratory chain or due to a combination of the mentioned effects.

On the basis of the fact that compound **10** in screenings assays was able to inhibit insulin release and depolarize mitochondrial membranes without affecting smooth muscle, we selected this compound for additional examination.

Effect of 10 on Perifused Rat Pancreatic Islets. ^{86}Rb Outflow. Compound **10** (10 μM) provoked a rapid and sustained increase in ^{86}Rb outflow from pancreatic islets perifused throughout in the presence of 5.6 mM glucose (Figure 2). The withdrawal of the drug from the perifusate was followed by an immediate reduction in ^{86}Rb outflow rate, indicating that the cationic response to the compound did not result from a damage of pancreatic islet cells. When the perifusate was enriched with the hypoglycaemic sulfonylurea glibenclamide (10 μM), the stimulatory effect of **10** was markedly reduced (Figure 2).

^{45}Ca Outflow and Insulin Release. In the presence of 16.7 mM glucose and extracellular Ca^{2+} in the perifusing medium, the addition of **10** (10 μM) elicited an immediate and sustained inhibition of both ^{45}Ca outflow and insulin release (Figure 3, upper and lower panel). During the period of exposure to **10** (60th−68th min), the release of insulin represented 11.81 ± 0.72% ($P < 0.05$) of that recorded before the administration of the drug (40^{th}-44^{th} min). The removal of **10** from the perifusate was accompanied by a slow increase in both ^{45}Ca outflow and insulin output (Figure 3). When the same experiment was repeated in the absence of extracellular Ca^{2+}, the basal rate of ^{45}Ca outflow (40^{th}-44^{th} min) was lower ($P < 0.05$). Under the latter experimental condition, the addition of **10** (10 μM) provoked a modest but sustained and reversible rise in the ^{45}Ca outflow rate (Figure 3, upper panel). In islets exposed throughout to external Ca^{2+} but in the absence of glucose, **10** (10 μM) again provoked a slight increase in ^{45}Ca outflow (data not shown).

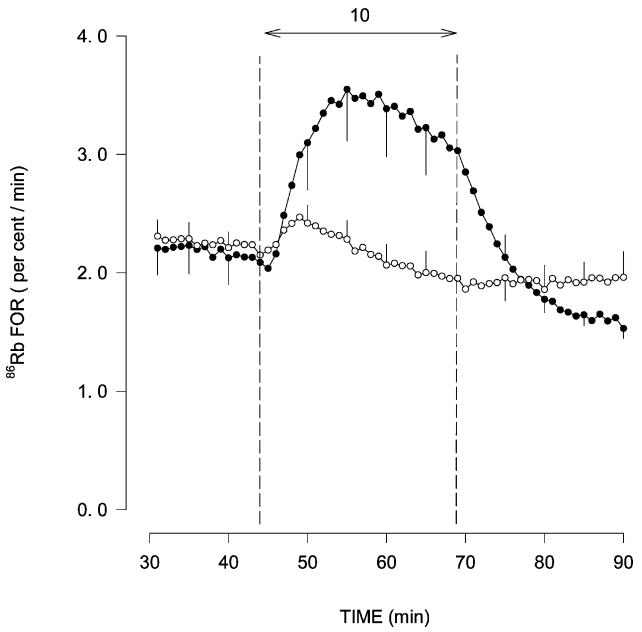


Figure 2. Effect of 10 μM **10** on ^{86}Rb outflow from rat pancreatic islets perifused throughout in the absence (●) or presence (○) of 10 μM glibenclamide. Basal media contained extracellular Ca^{2+} (2.56 mM) and glucose (5.6 mM). Mean values (± SE mean) refer to six individual experiments.

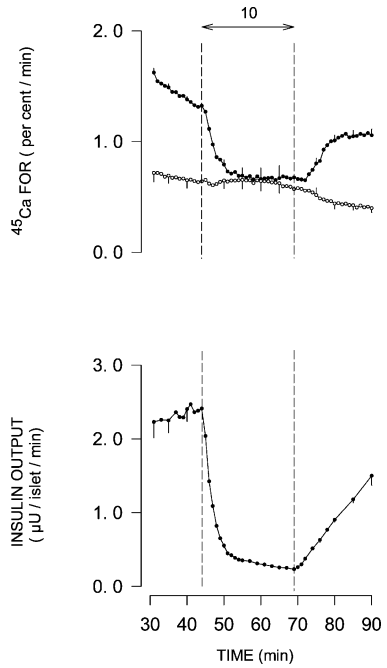


Figure 3. Effects of compound **10** on Ca^{2+} outflow and insulin release from isolated pancreatic islets. Mean values (± SE mean) refer to six individual experiments. Upper panel: Effect of 10 μM **10** on ^{45}Ca outflow from rat pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+} (●, 2.56 mM) or were deprived of Ca^{2+} and enriched with EGTA (○, 0.5 mM). Lower panel: Effect of 10 μM **10** on insulin release from rat pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+} (●, 2.56 mM).

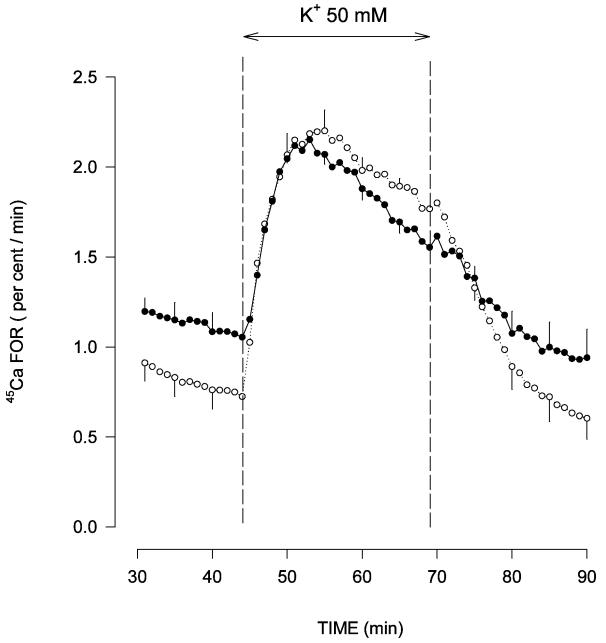


Figure 4. Effect of a rise in the extracellular concentration of K^+ from 5 to 50 mM on ^{45}Ca outflow from rat pancreatic islets perifused throughout in the absence (○) or presence of 10 μM **10** (●). Basal media contained extracellular Ca^{2+} (2.56 mM) and glucose (2.8 mM). Mean values (± SE mean) refer to five individual experiments.

In the last series of radio isotopic experiments, we examined the effect of **10** (10 μM) on the KCl-induced changes in ^{45}Ca outflow. A rise in the extracellular concentration of K^+ from 5 to 50 mM provoked a rapid and marked increase in ^{45}Ca FOR from pancreatic islet perifused in the presence of 2.8 mM glucose and extracellular Ca^{2+} (Figure 4). When the same experiments were repeated in the presence of **10** (10 μM) throughout, the basal rate of ^{45}Ca outflow (40th−44th min) was higher. Thus, ^{45}Ca outflow before the increase in extracellular K^+ averaged 0.75 ± 0.10%/min in the absence and 1.08 ± 0.11%/min in the presence of 10 μM **10** ($P < 0.1$), respectively. The presence of **10** (10 μM) in the basal medium failed, however, to counteract the cationic response to high extracellular K^+ concentration (Figure 3).

The radio isotopic experiments clearly revealed that the addition of micromolar concentrations of **10** provoked a rapid, pronounced and sustained increase in ^{86}Rb outflow from prelabeled and perifused rat pancreatic islets. Although the measurement of ^{86}Rb fractional outflow rate underestimates the real changes in K^+ fluxes,[29] such data suggest that the drug increases the membrane K^+ permeability.[27,29,30]

The K^+ channel activation mediated by **10** may be expected to hyperpolarize the beta cell plasma membrane and, in turn, inhibit the L-type voltage-dependent Ca^{2+} channels, reduce the Ca^{2+} inflow and, ultimately, inhibit the secretory process.

This proposed mechanism of action is attested by the finding that **10** induced a marked reduction in ^{45}Ca outflow and insulin release from rat pancreatic islets perifused in the presence of 16.7 mM glucose and extracellular Ca^{2+}. In islets exposed throughout to Ca^{2+} and insulinotropic concentrations of glucose, the ^{45}Ca fractional outflow rate is known to reflect a sustained stimulation of isotopic exchange between influent ^{40}Ca and effluent ^{45}Ca.[31] Thus, the inhibitory effect of **10** on ^{45}Ca outflow can be viewed as the result of a reduction of Ca^{2+} entry into the islet cells. In agreement with such a proposal, the decrease in ^{45}Ca outflow mediated by **10** did not occur when the islets were exposed to noninsulinotropic glucose concentrations or to Ca^{2+} deprived media.

The data from the radio isotopic experiments indirectly suggest that the drug could be a potent activator of K_{ATP} channels. Indeed, the enhancing effect of **10** on ^{86}Rb outflow is reminiscent of that evoked by other K_{ATP} channel openers[27,29,30] and is sensitive to glibenclamide, a hypoglycaemic sulfonylurea known to close K_{ATP} channels.[32,33]

All this experimental evidence indicates that the inhibitory effect of **10** on the insulin releasing process results from K_{ATP} channel activation. The failure of **10** to counteract the increment in ^{45}Ca outflow mediated by a high K^+ concentration (50 mM) further supports the hypothesis that the primary effect of the compound is to raise the K^+ permeability of the pancreatic beta cell. Indeed, this radioisotopic response to 50 mM K^+, which is mediated by the opening of L-type voltage-dependent Ca^{2+} channels, is known to be sensitive to Ca^{2+} entry blockers but resistant to K^+_{ATP} channel openers.[27,29−31]

Last, it should be noted that **10**, unlike other K_{ATP} channel openers,[4,27,29,30] elicited a glibenclamide-resistant modality of ^{86}Rb extrusion. Indeed, the hypoglycaemic sulfonylurea markedly reduced but failed to totally abolish the capacity of **10** to increase ^{86}Rb outflow from prelabeled and perifused rat pancreatic islets. Moreover, the drug provoked an increase in ^{45}Ca outflow from islets perifused in the absence or the presence of noninsulinotropic glucose concentrations. An identical pattern was revealed in islet exposed to 16.7 mM glucose and Ca^{2+}-deprived media. These findings suggest that **10** could promote intracellular calcium redistribution with subsequent changes in ^{45}Ca outflow or activation of a Ca^{2+}-sensitive but glibenclamide-resistant modality of ^{86}Rb extrusion.[34]

Electrophysiology. To further explore its mechanism of action, **10** was studied using the patch clamp technique. We have previously described that certain phenyl cyanoguanidines increase ion current flow through recombinant Kir6.2/SUR1 channels.[21] Compound **10** gave reproducible activation of whole-cell ion currents through Kir6.2/SUR1 channels expressed in HEK293 cells and in patch clamp experiments on Kir6.2/SUR1 expressed in *Xenopus* oocytes using the inside out configuration (Figure 5). In accordance with the lack of effect on phenylephrine-contracted aorta rings, compound **10** did not activate the Kir6.2/SUR2B channels expressed in Xenopus oocytes. In contrast, it was found (Figure 6) that the compound dose dependently inhibits the currents through metabolically activated (3 mM Na-azide) Kir6.1/SUR2B channels, indicating that the structural modifications (3-pyridyl to 3,5-bis(trifluormethyl)phenyl and 2-(3,3-dimethyl)butyl to cyclobutyl) have changed the efficacy of the cyanoguanidine derivative from an selective activator of Kir6.2/SUR2 K_{ATP} channels to an activator of Kir6.2/SUR1 and a blocker of Kir6.2/SUR2B channels.

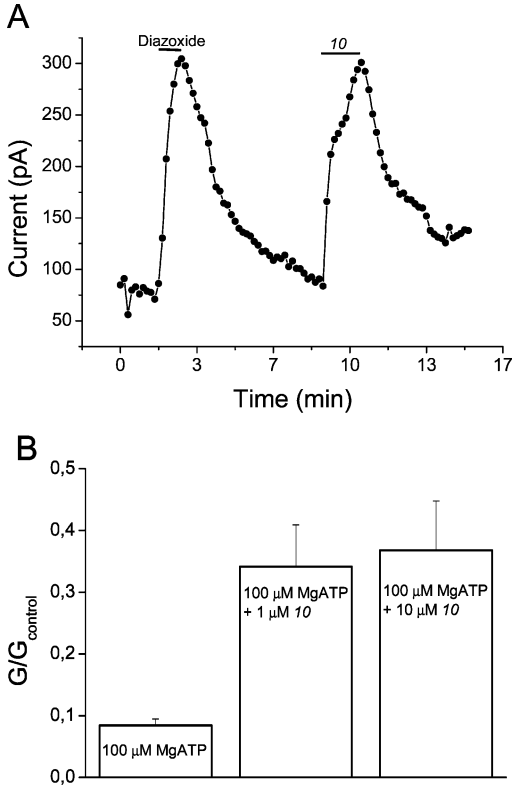


Figure 5. Effects of **10** on Kir6.2/SUR1 channels. A: Whole cell recording from an HEK293 cell expressing Kir6.2/SUR1. The points indicate the current obtained in response to a 10 mV depolarizing pulse applied every 10 s from a holding potential of −80 mV. Diazoxide (300 μM) and **10** (10 μM) were applied as indicated by the horizontal bars. B: Effect of **10** on macroscopic currents through Kir6.2/SUR1 channels in inside-out patches. Mean macroscopic conductances (G) are expressed as a fraction of the mean slope conductance in nucleotide and drug free solution ($G_{control}$). Data were obtained in the presence of 100 μM MgATP, 100 μM MgATP plus 1 μM **10**, or 100 μM MgATP plus 10 μM **10**.

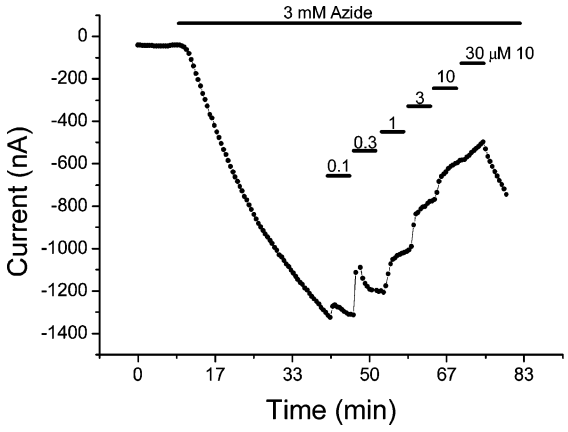


Figure 6. Effects of **10** on Kir6.2/SUR2B channels. Representative time course of 10 induced block of Kir6.2/SUR2B current activated by Na-azide. Currents were evoked by 10 mV hyperpolarizing steps from a holding potential of −10 mV every 30 s.

Conclusion

N-Aryl-cyanoguanidines have been optimized to get compounds, e.g. **10**, which, in vitro, inhibit glucose-stimulated insulin release. Compound **10** activates Kir6.2/SUR1 channels of beta cells and depolarize mitochondrial membranes through an unknown uncoupling mechanism or an activation of mitoK_{ATP} channels. The ability of **10** to inhibit glucose-induced insulin release is presumably mediated through a modulation of intracellular Ca^{2+} concentrations caused by activation of Kir6.2/SUR1 K_{ATP} channels or depolarization of mitochondrial membranes, which will mobilize intracellular Ca^{2+} stores. The combined effects of inducing beta cell rest and deactivating mitochondria could improve the ability of this compound to protect beta cells against metabolic and toxic stress. A detailed presentation of the beta cell protective effects of **10** will be published (Sandler et al.).

Compound **10** was furthermore found to inhibit ion currents through the Kir6.2/SUR2B K_{ATP} channels of smooth muscles. This supports previous findings that structural modifications of selective K_{ATP} channel openers such as pinacidil could change the K_{ATP} channel efficacies and that it therefore is possible to identify compounds which are activators of one K_{ATP} channel subtype and blockers of another.[28,35,36]

Experimental Section

Chemistry. Reagents, starting materials and solvents were purchased from common commercial suppliers and were used as received. All dry solvents were dried overnight over molecular sieves (0.3 or 0.4 nm). Evaporation was carried out on a rotary evaporator at bath temperatures <40 °C and under appropriate vacuum. Flash chromatography was carried out on a Biotage flash 40 using Biotage flash columns (KP−SIL, 60 Å particle size 32−63 μm). Melting points were determined with a Büchi B545 apparatus and are uncorrected. Proton NMR spectra were recorded at ambient temperature using a Brucker Avance DPX 200 (200 MHz), Brucker Avance DPX 300 (300 MHz), and Brucker Avance DPX 400 (400 MHz) with tetramethylsilane as an internal standard. Chemical shifts (δ) are given in ppm and splitting patterns are designated as follows: s, singlet; d, doublet; dd, double doublet; dt, double triplet t, triplet, tt, triplet of triplets; q, quartet; quint, quintet; sext, sextet; m, multiplet, and br = broad. The 70 eV EI solid mass spectra were recorded on a Finnigan MAT-TSQ 70 mass spectrometer. Liquid chromatography−mass spectrometry (LC-MS) analysis was performed on HP1100 MSD equipped with a Waters Xterra MS C-18 × 3 mm column. Reactions were followed by thin-layer chromatography performed on silica gel 60 F254 (Merck) or ALUGRAMSIL G/UV_{254} (MACHEREY-NAGEL) TLC aluminum sheets. Elemental analyses (C, H, N) were performed by Novo Nordisk, Microanalytical Laboratory, Denmark.

***N*-[3,5-Bis(trifluoromethyl)phenyl]-*N′*-cyano-*O*-phenylisourea (1a).** A solution of diphenylcyanocarbonimidate (2 mmol, 476 mg), 3,5-bis(trifluoromethyl)aniline (2 mmol, 458 mg), and triethylamine (2 mmol, 202 mg) in dichloromethane (15 mL) was stirred under nitrogen for 12 h. After concentration, the residue was stirred with toluene (5 mL) for 2 h and the solid was collected by filtration giving 550 mg of the title compound (73.6%); mp 190.5−191.5 °C; EI SP/MS; ^{1}H NMR.

***N*-Cyano-*N′*-[3,5-bis(trifluoromethyl)phenyl]-*N″*-(*n*-propyl)guanidine (2). 1a** (0.94 mmol, 350 mg) and *n*-propylamine (1 mL) was stirred in a sealed flask for 19 h at 75 °C. After concentration, the residue was dissolved in dichloromethane, washed with 1 N aqueous HCl (2x), water, dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (ethyl acetate/heptane 1:2) to give the title compound (90 mg, 28%) as white crystals. Mp 142.5−143.5 °C; ^{1}H NMR. Anal. ($C_{13}H_{12}F_6N_4$) C, H, N.

***N*-Cyano-*N′*-[3,5-bis(trifluoromethyl)phenyl]-*N″*-(3-methylbutyl)guanidine (3).** A solution of **1a** (1 mmol, 373 mg), 3-methylbutylamine (1.15 mmol,100 mg) and triethylamine (1.5 mmol, 150 mg) in acetonitrile (2 mL) was stirred

for 24 h at 60 °C. After concentration, the residue was purified by column chromatography (heptane/ethyl acetate 1:1) to give the title compound (100 mg, 27%). ^{1}H NMR. Anal. ($C_{13}H_{12}F_6N_4$) C, H, N.

***N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-(1-isopropyl)guanidine (4).** **1a** (0.94 mmol, 350 mg) and isopropylamine (1 mL) was stirred in a sealed flask for 19 h at 75 °C. After concentration, the residue was dissolved in dichloromethane, washed with 1 N aqueous HCl (2×), and water, dried (Na_2SO_4), and concentrated. The residue was crystallized in ethyl acetate and heptane to give the title compound (114 mg, 45%) as white crystals. Mp 176−182 °C; ^{1}H NMR. Anal. ($C_{13}H_{12}F_6N_4$), C, H, N.

***N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-(1,2-dimethylpropyl)guanidine (5).** A solution of **1a** (0.8 mmol, 300 mg), 3-methyl-2-butylamine (0.88 mmol, 0.101 mL), and triethylamine (0.88 mmol, 0.123 mL) in acetonitrile (2 mL) was stirred for 7 h at 75 °C. After concentration, the residue was dissolved in ethyl acetate, washed with water, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (heptane/ethyl acetate 4:1) to give the title compound (143 mg, 59%) as white crystals. Mp 134−136 °C; EI SP/MS; ^{1}H NMR; Anal. ($C_{15}H_{16}F_6N_4$) C, H, N.

(*S*)-*N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-(1-methyl-2,2-dimethylpropyl)guanidine (6). To a solution of **1a** (1.0 mmol, 373 mg) in dry acetonitrile (3.5 mL) were added (*S*)-3,3-dimethyl-2-butylamine (1.1 mmol, 111 mg, 145 μL) and triethylamine (1.1 mmol, 111 mg, 153 μL). The mixture was stirred in a sealed flask for 16 h at 80 °C under nitrogen. After concentration, the residue was purified by flash chromatography (ethyl acetate/heptane 1:2) to give the title compound (152 mg, 40%) as white crystals. Mp 167.9−168.7 °C. Anal. ($C_{16}H_{18}F_6N_4$) C, H, N.

(*R*)-*N*-Cyano-*N*′-(3,5-bis(trifluoromethyl)phenyl)-*N*″-(1-methyl-2,2-dimethyl-propyl)guanidine (7). To a solution of **1a** (1.0 mmol, 373 mg) in dry acetonitrile (3.5 mL) were added (*R*)-3,3-dimethyl-2-butylamine (1.1 mmol, 111 mg, 145 μL) and triethylamine (1.1 mmol, 111 mg, 153 μL). The mixture was stirred in a sealed flask for 16 h at 80 °C under nitrogen. After concentration, the residue was purified by flash chromatography (ethyl acetate/heptane 1:3) to give the title compound (236 mg, 62%) as white crystals. Mp 167.9−168.7 °C; ^{1}H NMR. Anal. ($C_{16}H_{18}F_6N_4$) C, H, N.

***N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-(1,1-dimethylpropyl)guanidine (8).** A solution of **1a** (0.78 mmol, 290 mg), *tert*-amylamine (0.85 mmol, 0.100 mL), and triethylamine (0.85 mmol, 0.119 mL) in acetonitrile (2 mL) was stirred for 25 h at 75 °C followed by workup as described for **5** to give the title compound (80 mg, 28%) as white crystals. Mp 149−150 °C; EI SP/MS. ^{1}H NMR. Anal. ($C_{15}H_{16}F_6N_4 \cdot 0.15H_2O$) C, H, N.

***N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-[1-(3-chlorophenyl)cyclobut-1-yl]guanidine (9).** To a suspension of **1a** (1.5 mmol, 570 mg) and 1-(3-chlorophenyl)cyclobut-1-ylamine (1.8 mmol, 390 mg) in dry acetonitrile (5.0 mL) was added triethylamine (3.75 mmol, 379 mg, 525 μL). The mixture was refluxed for 14 h under nitrogen. After concentration, the residue was purified by flash chromatography using ethyl acetate/heptane 1:3 to give the title compound (153 mg, 22%) as white crystals. Mp 181.1−182.4 °C; ^{1}H NMR. Anal. ($C_{20}H_{15}ClF_6N_4$) C, H, N.

***N*-Cyano-*N*′-[3,5-bis(trifluoromethyl)phenyl]-*N*″-(cyclopentyl)guanidine (10).** To a suspension of **1a** (0.400 g, 1.1 mmol) in dry acetonitrile (2.5 mL) were added triethylamine (0.164 mL, 1.2 mmol) and cyclopentylamine (0.116 mL, 1.2 mmol). The homogeneous solution was stirred at 85 °C under N_2 for 3.5 h. The solvent was evaporated, and the residue was dissolved in ethyl acetate and washed with water twice. The organic layer was dried (Na_2SO_4) and concentrated. The crude product was purified by flash chromatography using ethyl acetate/heptane 1:2 to give the title compound. Yield 82% (0.320 g). mp 156−159 °C. EI SP/MS. ^{1}H NMR. Anal. ($C_{15}H_{14}F_6N_4 \cdot 0.2H_2O$) C, H, N.

***N*-Cyano-*N*′-[3-methoxy-5-(trifluoromethyl)phenyl]-*O*-phenylisourea (1b).** To a solution of 3-methoxy-5-trifluoromethylaniline (11 mmol, 2.10 g) in dichloromethane (25 mL) was added diphenyl cyanocarbonimidate (10 mmol, 2.38 g), and triethylamine (11 mmol, 1.53 mL). The reaction mixture was stirred under nitrogen for 16 h at room temperature. After concentration, the residue was stirred with water and then the water was decanted off. The residue was purified by flash chromatography (ethyl acetate/heptane 1:2) to give 0.413 g of the title compound (12%); Mp 168.5−169.5°C. EI SP/MS; ^{1}H NMR.

***N*-Cyano-*N*′-cyclopentyl-*N*″-[3-methoxy-5-(trifluoromethyl)phenyl]guanidine (11).** To a solution of **1b** (0.52 mmol, 175 mg) in dry acetonitrile (1 mL) were added cyclopentylamine (1.04 mmol, 0.113 mL) and triethylamine (0.080 mL). The mixture was stirred for 19 h at 75 °C under nitrogen. After concentration, the residue was dissolved in dichloromethane, washed with 1 N aqueous HCl (2×), and water, dried (Na_2SO_4), and concentrated. The residue was purified by flash chromatography (ethyl acetate/heptane 1:2) to give a syrup. Crystallization in ethyl acetate/heptane 1:4 gave gray crystals (110 mg, 65%). Mp 101−102 °C; EI SP/MS; ^{1}H NMR. Anal. ($C_{15}H_{17}F_3N_4O$) C, H, N.

***N*-Cyano-*N*′-(3,5-dichlorophenyl)-*O*-phenylisourea (1c).** To a solution of 3,5-dichloroaniline (11 mmol,1.79 g) in dichloromethane (25 mL) were added diphenylcyanocarbonimidate (10 mmol, 2.38 g) and triethylamine (11 mmol, 1.53 mL). The reaction mixture was stirred under nitrogen for 65 h at room temperature. After concentration, the residue was stirred with water and the water was decanted off. The residue was stirred with toluene, and the solid was collected by filtration, giving 2.04 g of the title compound (67%). EI SP/MS; ^{1}H NMR.

***N*-Cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(3-methylbutyl)-guanidine (12).** To **1c** (0.98 mmol, 300 mg) in dry acetonitrile (2 mL) were added 3-methylbutylamine (2.16 mmol, 0.255 mL) and triethylamine (1.08 mmol, 0.150 mL). The reaction mixture was stirred for 16 h at 85 °C under nitrogen. The precipitated material was filtered off and recrystallized from ethyl acetate to give the title compound (160 mg, 54%) as white crystals. Mp 146.5−151.5 °C; ^{1}H NMR. Anal. ($C_{13}H_{16}Cl_2N_4$) C, H, N.

***N*-Cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(1-isopropyl)guanidine (13). 1c** (0.98 mmol, 300 mg) and isopropylamine (1 mL) were stirred in a sealed flask for 19 h at 75 °C. After concentration, the residue was dissolved in dichloromethane, washed with 1 N aqueous HCl (2×) and water, dried (Na_2SO_4), and concentrated. The residue was crystallized in ethyl acetate/heptane 1:3 to give the title compound (115 mg, 43%) as white crystals. Mp 156−158.5 °C; ^{1}H NMR. Anal. ($C_{11}H_{12}Cl_2N_4$) C, H, N.

(*S*)-*N*-Cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(1-phenylethyl)guanidine (14). A solution of **1c** (1.3 mmol, 400 mg), triethylamine (1.4 mmol, 0.200 mL), and (*S*)-1-phenylethylamine (1.4 mmol, 0.185 mL) in acetonitrile (3.5 mL) was stirred in a sealed flask for 18 h at 80 °C. The cooled reaction mixture was concentrated, and the residue was purified by flash chromatography using ethyl acetate/heptane 1:3 as eluent to give the title compound (269 mg, 63%) as a clear oil. LC/MS; ^{1}H NMR; Anal. ($C_{16}H_{14}Cl_2N_4$) C, H, N.

(*R*)-*N*-Cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(1-phenylethyl)guanidine (15). A solution of **1c** (1.3 mmol, 400 mg), triethylamine (1.4 mmol, 0.200 mL), and (*R*)-1-phenylethylamine (1.4 mmol, 0.183 mL) in acetonitrile (3.5 mL) was stirred in a sealed flask for 18 h at 80 °C. The cooled reaction mixture was concentrated, and the residue was purified by flash chromatography using ethyl acetate/heptane 1:3 as eluent to give the title compound (218 mg, 51%) as a foam. LC/MS; ^{1}H NMR. Anal. ($C_{16}H_{14}Cl_2N_4$) C, H, N.

***N*-Cyano-*N*′-(3,5-dichlorophenyl)-*N*″-(1,1-dimethylpropyl)guanidine (16).**[22] To a solution of **1c** (0.8 mmol, 250 mg) in dry acetonitrile (2 mL) were added *tert*-amylamine (0.9 mmol, 0.105 mL) and triethylamine (0.125 mL). The mixture was stirred for 20 h at 70°C under nitrogen. After concentra-

tion, the residue was dissolved in ethyl acetate, washed with water (2×), dried (Na_2SO_4), and concentrated. The residue was purified by flash chromatography (ethyl acetate/heptane 1:4) to give the title compound (158 mg, 65%) as crystals. Mp 158.5−160°C (lit.[22] 151−153 °C); EI SP/MS; ^{1}H NMR. Anal. ($C_{13}H_{16}Cl_2N_4$) C, H, N.

***N*-Cyano-*N′*-cyclopentyl-*N″*-(3,5-dichlorophenyl)guanidine (17).** To a solution of **1c** (0.8 mmol, 250 mg) in dry acetonitrile (2 mL) were added cyclopentylamine (0.9 mmol, 0.089 mL) and triethylamine (0.125 mL). The mixture was stirred for 2.5 h at 82 °C under nitrogen. After concentration, the residue was purified by flash chromatography (ethyl acetate/heptane 1:2) to give the title compound (160 mg, 66%) as crystals. Mp 147.5−148.5 °C; EI SP/MS; ^{1}H NMR. Anal. ($C_{13}H_{14}Cl_2N_4$) C, H, N.

***N*-Cyano-*N′*-(3,5-difluorophenyl)-*O*-phenylisourea (1d).** To a solution of 3,5-difluoroaniline (11 mmol, 1.42 g) in dichloromethane (25 mL) were added diphenylcyanocarbonimidate (10 mmol, 2.38 g) and triethylamine (11 mmol, 1.53 mL). The reaction mixture was stirred under nitrogen for 65 h at room temperature. After concentration in vacuo at 45 °C, the residue was stirred with toluene. The title compound was isolated as a white powder by filtration of the precipitate. Yield 1.63 g (60%); Mp 184−188 °C; EI SP/MS; ^{1}H NMR.

***N*-Cyano-*N′*-(3,5-difluorophenyl)-*N″*-(3-methylbutyl)-guanidine (18).** A solution of **1d** (1.5 mmol, 0.40 g) in dry acetonitrile (2.5 mL) were added 3-methylbutylamine (1.6 mmol, 0.19 mL) and triethylamine (1.6 mmol, 0.225 mL). The reaction mixture was stirred for 18 h at 85 °C under nitrogen. After concentration, the residue was stirred with water. Filtration and recrystallization of the precipitate from ethyl acetate/heptane 1:2 gave the title compound. Yield 0.290 g (75%). Mp 118°-122 °C; EI SP/MS; ^{1}H NMR. Anal. ($C_{13}H_{16}F_2N_4$) C, H, N.

***N*-Cyano-*N′*-(3-methylbutyl)-*N″*-(3-pyridyl)guanidine (19).** To a solution of 3-aminopyridine (2.3 mmol, 0.215 g) in dichloromethane (7.5 mL) were added diphenylcyanocarbonimidate (2.5 mmol, 0.60 g) and triethylamine (2.5 mmol, 0.359 mL). The reaction mixture was stirred under nitrogen for 17 h at room temperature. After concentration, the residue was stirred with water, the water was decanted off. The residue was crystallized in toluene to give 0.459 g *N*-cyano-*O*-phenyl-*N′*-(3-pyridyl)isourea (77%); Mp 123−125 °C; EI SP/MS; 238 (M^+). ^{1}H NMR (CDCl3): δ 7.13 (d, 2H), 7.33 (m, 2H), 7.40 (t, 2H), 7.75 (br d, 1H), 8.48 (d, 1H), 8.70 (br s, 1H), 9.0 ppm (br, 1H).

To a solution of *N*-cyano-*O*-phenyl-*N′*-(3-pyridyl)isourea (1.5 mmol, 0.35 g) in dry acetonitrile (3 mL) was added triethylamine (1.6 mmol, 0.22 mL) and 3-methylbutylamine (1.6 mmol, 0.19 mL). The mixture was stirred for 35 h at 80 °C under nitrogen. After concentration, the residue was purified by flash chromatography (ethyl acetate/methanol 9:1) followed by recrystallization to give the title compound (0.13 g, 38%) as white crystals. Mp 136.5−137 °C; EI SP/MS: ^{1}H NMR. Anal. ($C_{12}H_{17}N_5$) C, H, N.

Biology. Effects on membrane potential in βTC3 cells, inhibition of glucose-stimulated insulin release in vitro in βTC6 cells isolated or rat islets (static incubations) and relaxation of phenylephrine-contracted rat aortic rings in vitro were determined according to procedures described in Nielsen et al.[4]

Mitochondrial Membrane Potential. The βTC3 cells were seeded out in 96-well plates 2 days prior to experiments at 50 000 cells per well. They were cultured in DMEM cell culture medium supplemented with 1000 mg/L glucose, 10% FCS, penicillin, and streptomycin. At the day of experiment, loading buffer (KRW buffer (containing in mM: NaCl (140), KCl (3.6), NaH_2PO_4 (0.5), $MgSO_4$ (0.5), $NaHCO_3$ (2.0), $CaCl_2$ (1.5), HEPES (10), pH 7.4 (adjusted with NaOH, 1 M) supplemented with 5 μM JC-1 (Molecular Probes, Leiden, The Netherlands, T-3168) and containing 10% FCS) prewarmed to 37 °C was added to all wells still containing culture medium (100 μL in each).

Plates were then incubated at 37 °C, 5.0% CO_2, for 15 min. The loading buffer was removed, and the cells were washed once with 100 μL of KRW buffer preheated to 37 °C. Finally, 160 μL of KRW (supplemented with 2.5 mM glucose) preheated to 37 °C was added to all wells, and the plate was placed in a NovoStar fluorescence plate reader together with a polypropylene plate (Greiner 650201) used to plate out 5× concentrated dilution series of test compounds in KRW containing 0.5% DMSO (starting at the highest concentration of the of the test compound at a 1:3 dilution). Compounds were tested as multiple dilution series with 8 points in each. After 15 min., an initial reading was taken from the plate. Compounds were then added manually from the compound plate with a 12-channel multipipet (40 μL/well) and the assay incubated for 30 s, 5 min, and 10 min. A second reading was taken using the same measurement protocol without any adjustment of the signal gains after 30 s, 5 min, and 10 min.

The assay on A10 smooth muscle cells were made with a similar protocol, however loaded in a 50/50 mixture of KRW and loading buffer and measured in a FLIPR plate reader which is a more sensitive system than the NovoStar, and the measurements were solely based on the green fluorescence from JC-1.

Measurements of ^{86}Rb, ^{45}Ca Outflow and Insulin Release from Perifused Rat Pancreatic Islets. Experiments were performed with pancreatic islets isolated by the collagenase method from fed Wistar rats. The methods used to measure ^{86}Rb (^{42}K substitute) outflow, ^{45}Ca outflow, and insulin release from perifused islets have been described previously.[30,31] Groups of 100 islets were incubated for 60 min in a bicarbonate-buffered medium (in mM: 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$) containing 16.7 mM glucose and either ^{86}Rb ion (0.15−0.25 mM; 50 μCi mL^{-1}) or ^{45}Ca ion (0.02−0.04 mM; 100 μCi mL^{-1}). After incubation, the islets were washed four times with a nonradioactive medium and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL min^{-1}). From the 31st to the 90th min of perifusion, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.5 mL) was used for scintillation counting while the remainder was stored at −20 °C for insulin radioimmunoassay.[37] At the end of the perifusion, the radioactive content of the islets was also determined. The outflow of ^{86}Rb or ^{45}Ca ion (cpm min^{-1}) was expressed as a fractional outflow rate (% of instantaneous islet content min^{-1}, FOR).

According to the experiments, the media were enriched with glucose (Merck, Darmstadt, Germany), glibenclamide (ICN Biomedicals, Inc., OH) or **10**. Glibenclamide and **10** were dissolved in dimethylsulfoxide which was added to both control and test media. At the final concentrations used, dimethyl sulfoxide fails to affect islet function.[29,30] When high concentrations of extracellular K^+ were used, the concentration of extracellular NaCl was lowered to keep osmolarity constant.

Results are expressed as the mean (± SEM). The statistical significance of the differences between mean data was assessed by use of Student's *t*-test.

Measurements of Glucose Oxidation in Human Hepatocyte Cell Line HEP-G2. The assay measures indirectly the activity of the respiratory chain in HEP-G2 cells by using d-(5-^{3}H(N))-glucose. Upon oxidation, the ^{3}H-proton will be incorporated into water. The water is thereafter separated from the D-(5-^{3}H(N))-glucose by evaporation. Finally, the radioactivity in the water is determined using a Topcounter. Cells are seeded at a concentration of 2 × 104 cells/100 μL/ well and then incubate at 37 °C and 5% CO_2 overnight.

The next day the compounds to be tested are diluted in DMSO (Sigma, St. Louis, MO) to 100 times final concentration, and then they are diluted to final concentration in assay medium containing 10 μCi/mL D-(5-^{3}H(N))-glucose (Perkin-Elmer Life Sciences Inc., Boston, MA). The medium is removed from the cells, and 200 μL of the compounds dilutions are added in duplicates. The cells are then incubated for another 3 h at 37 °C and 5% CO_2. Finally the cells are lysed by adding 50 μL of 10% TCA (trichloroacetate), and the radioactive water

is thereafter separated from the D-(5-^{3}H(N))-glucose by evaporation. The results are expressed in mean ± SEM of three separate experiments.

Mitochondria Respiration. Mitochondria were prepared according to standard procedure[36] from Sprague−Dawley male rats (approximately 250 g) in a medium containing 250 μM sucrose, 5 mM HEPES, and 1 mM EDTA (pH 7.2). Mitochondrial state IV respiration rate was measured using a Clark-type oxygen electrode. Mitochondria were incubated at 37 °C in a total volume of 10 mL containing 1.6 mg mitochondrial protein/mL in medium containing 220 mM D-mannitol, 5 mM $MgCl_2$, 2 mM HEPES, and 5 mM K_3PO_4 (pH 7.4) in the presence of 10 mM succinic acid, 2.5 μM rotenone, and 1 μg/mL oligomycin.

Electrophysiology. Mammalian Cells. Whole cell currents were recorded from HEK293 cells stably expressing human Kir6.2/SUR1 channels, using an EPC9 patch clamp amplifier (HEKA Electronic GmbH, Lambrecht, Germany). Cells were clamped at −70 mV and currents evoked by repetitive 250 ms, 10 mV depolarizing voltage steps. Currents were filtered at 2 kHz and sampled at 10 kHz. The internal solution contained (in mM): 120 KCl, 1 $MgCl_2$, 5 EGTA, 2 $CaCl_2$, 20 HEPES, 0.3 MgADP, 5 MgATP (pH 7.3). The external solution was (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 mannitol, 10 HEPES (pH 7.2 with NaOH).

Oocytes. Oocyte collection and mRNA injection Female *Xenopus laevis* were anaesthetized with MS222 (2 g/L added to the water). From each animal was removed one ovary via a minilaparotomy, and the incision was sutured and the animal allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation, and the animal was then killed by decapitation while under anaesthesia. Oocytes were coinjected with about 0.2 ng of Kir6.2 (Genbank D50582) and about 2 ng of mRNA encoding SUR2B (Genbank AF061324). The final injection volume was 50 nL/oocyte. Isolated oocytes were maintained in Barth's solution and manually defollicated after 30 min collagenase (Sigma type V) incubation. Injected oocytes were studied 3 to 7 days after injection.

Xenopus Oocyte Electrophysiology: Two-Electrode Voltage Clamp (TEVC). Whole-cell currents were recorded from oocytes expressing Kir6.2/SUR2B at 20 to 24 °C using a two-electrode voltage-clamp amplifier (Warner OC725) and analyzed using in-house software.[24] Currents were filtered at 0.1 kHz and digitized at 0.24 kHz. TEVC electrodes were pulled from thin-walled borosilicate glass and had resistances between 0.4 and 1 MΩ when filled with 3 mol/L KCl. K_{ATP} currents were activated by metabolic inhibition with 3 mmol/L azide, and currents were recorded in extracellular solution containing (mmol/L): KCl 90, MgCl2 2.5, HEPES 10 (pH 7.2 with KOH). The holding potential was set to the zero current potential (−10 or −20 mV). Hyperpolarizations of 10 or 20 mV amplitude of 3 s duration were applied every 30 s. The test compounds were prepared as 200 mmol/L stock solutions in DMSO. In control experiments, the maximal DMSO concentration applied (0.15%) was without effect on the K_{ATP} current.

Xenopus Oocyte Electrophysiology: Macropatches. Currents were recorded from giant inside-out patches excised from oocytes expressing Kir6.2/SUR1 channels using an EPC7 patch-clamp amplifier. Currents were evoked by repetitive 3 s voltage ramps from −110 mV to +100 mV, filtered at 0.2 kHz, and digitized at 0.4 kHz. The external (pipet) 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, 2 $MgCl_2$, 1 $CaCl_2$ 10 EGTA, 10 HEPES (pH 7.2 with KOH); final [K^+]: 140 mM). The slope conductance (G) was measured by fitting a straight line to the current−voltage relation between −20 mV and −100 mV. Drug effects were calculated as the conductance in the presence of drug (G) relative to the conductance in drug and nucleotide-free solution (G_c).

Acknowledgment. We thank Tinna Fremming, Anette Kock, Ann-Charlott Nielsen, Jette Møller, Vibeke Nielsen, and Berit Gerlach for technical assistance.

Supporting Information Available: Spectroscopic data and elementary analysis for compounds **1a**−**d**, **2**−**19**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Seino, S.; Miki, T. Physiological and Pathophysiological Roles of ATP−Sensitive K+ Channels. *Prog. Biophys Mol. Biol.* **2003**, *81*, 133−176.
(2) Ashcroft, F. M.; Gribble, F. M. ATP−Sensitive K+ Channels and Insulin Secretion: Their Role in Health and Disease. *Diabetologia* **1999**, *42*, 903−919.
(3) Seino, S. ATP−Sensitive Potassium Channels: a Model of Heteromultimeric Potassium Channel/Receptor Assemblies. *Annu. Rev. Physiol.* **1999**, *61*, 337−362.
(4) Nielsen, F. E.; Bodvarsdottir, T. B.; Worsaae, A.; MacKay, P.; Stidsen, C. E.; Boonen, H. C. M.; Pridal, L.; Arkhammar, P. O. G.; Wahl, P.; Ynddal, L.; Junager, F.; Dragsted, N.; Tagmose, T. M.; Mogensen, J. P.; Koch, A.; Treppendahl, S. P.; Hansen, J. B. 6-Chloro-3-alkylamino-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-Dioxide Derivatives Potently and Selectively Activate ATP Sensitive Potassium Channels of Pancreatic Beta-Cells. *J. Med. Chem.* **2002**, *45*, 4171−4187.
(5) Lebrun, P.; Devreux, V.; Hermann, M.; Herchuelz, A. Pinacidil Inhibits Insulin Release by Increasing K+ Outflow From Pancreatic B−Cells. *Eur. J. Pharmacol.* **1988**, *156*, 283−286.
(6) Dabrowski, M.; Ashcroft, F. M.; Ashfield, R.; Lebrun, P.; Pirotte, B.; Egebjerg, J.; Hansen, J. B.; Wahl, P. The Novel Diazoxide Analogue 3-Isopropylamino-7-Methoxy-4H-1,2,4-Benzothiadiazine 1,1-Dioxide Is a Selective Kir6.2/SUR1 Channel Opener. *Diabetes* **2002**, *51*, 1896−1906.
(7) Hansen, A. M. K.; Christensen, I. T.; Hansen, J. B.; Carr, R. D.; Ashcroft, F. M.; Wahl, P. Differential Interactions of Nateglinide and Repaglinide on the Human Beta-Cell Sulphonylurea Receptor 1. *Diabetes* **2002**, *51*, 2789−2795.
(8) Inoue, I.; Nagase, H.; Kishi, K.; Higuti, T. ATP−Sensitive K+ Channel in the Mitochondrial Inner Membrane. *Nature* **1991**, *352*, 244−247.
(9) Garlid, K. D. Mitochondrial Volume Control. In *Integration of Mitochondrial Function*; Lemasters, J. J., Hackenbrock, C. R., Thurman, R. G., and Westerhoff, H. V., Eds.; Plenum Publishing: New York, 1988; pp 257−276.
(10) Grover, G. J.; Garlid, K. D. ATP−Sensitive Potassium Channels: a Review of Their Cardioprotective Pharmacology. *J. Mol. Cell. Cardiol.* **2000**, *32*, 677−695.
(11) Garlid, K. D.; Paucek, P.; Yarov-Yarovoy, V.; Murray, H. N.; Darbenzio, R. B.; D'Alonzo, A. J.; Lodge, N. J.; Smith, M. A.; Grover, G. J. Cardioprotective Effect of Diazoxide and Its Interaction With Mitochondrial ATP−Sensitive K+ Channels. Possible Mechanism of Cardioprotection. *Circ. Res.* **1997**, *81*, 1072−1082.
(12) Krenz, M.; Oldenburg, O.; Wimpee, H.; Cohen, M. V.; Garlid, K. D.; Critz, S. D.; Downey, J. M.; Benoit, J. N. Opening of ATP−Sensitive Potassium Channels Causes Generation of Free Radicals in Vascular Smooth Muscle Cells. *Basic Res. Cardiol.* **2002**, *97*, 365−373.
(13) Jilkina, O.; Kuzio, B.; Grover, G. J.; Kupriyanov, V. V. Effects of K(ATP) Channel Openers, P-1075, Pinacidil, and Diazoxide, on Energetics and Contractile Function in Isolated Rat Hearts. *J. Mol. Cell. Cardiol.* **2002**, *34*, 427−440.
(14) Kowaltowski, A. J.; Seetharaman, S.; Paucek, P.; Garlid, K. D. Bioenergetic Consequences of Opening the ATP−Sensitive K(+) Channel of Heart Mitochondria. *Am. J. Physiol. Heart. Circ. Physiol.* **2001**, *280*, H649−H657.
(15) Alemzadeh, R.; Slonim, A. E.; Zdanowicz, M. M.; Maturo, J. Modification of Insulin Resistance by Diazoxide in Obese Zucker Rats. *Endocrinology* **1993**, *133*, 705−712.
(16) Rasmussen, S. B.; Sorensen, T. S.; Hansen, J. B.; Mandrup-Poulsen, T.; Hornum, L.; Markhoist, H. Functional Rest Through Intensive Treatment With Insulin and Potassium Channel Openers Preserves Residual Beta-Cell Function and Mass in Acutely Diabetic BB Rats. *Hormone Met.* **2000**, *32*, 294−300.
(17) Carr, R. D.; Brand, C. L.; Bödvarsdottir, T. B.; Hansen, J. B.; Sturis. J. NN414, a SUR1/Kir6.2-Selective Potassium Channel Opener, Reduces Blood Glucose, Improves Glucose Tolerance in the VDF Zucker Rat. *Diabetes* **2003**, *52*, 2513−2518.
(18) Maedler, K.; Sturis, J.; Lehmann, R.; Spinas, G. A.; Donath. M. Y. The SUR1/Kir6.2 Selective Potassium Channel Opener NN414 Prevents Glucose-Induced Apoptosis in Human Pancreatic β-Cells. *Diabetes* **2002**, *51*, A386.
(19) Kullin, M.; Li, Z. C.; Hansen, J. B.; Bjork, E.; Sandler, S.; Karlsson, F. A. K-ATP Channel Openers Protect Rat Islets Against the Toxic Effect of Streptozotocin. *Diabetes* **2000**, *49*, 1131−1136.

(20) Kullin, M.; Li, Z.; Hansen, J. B.; Welsh, N.; Karlsson, F. A.; Sandler, S. Protection of Rat Pancreatic Islets by Potassium Channel Openers Against Alloxan, Sodium Nitroprusside and Interieukin-1 Beta Mediated Suppression − Possible Involvement of the Mitochondrial Membrane Potential. *Diabetologia* **2003**, *46*, 80−88.
(21) Tagmose, T. M.; Mogensen, J. P.; Agerholm, P. C.; Arkhammar, P. O. G.; Wahl, P.; Worsaae, A.; Hansen, J. B. Phenylcyanoguanidines As Inhibitors of Glucose-Induced Insulin Secretion From Beta Cells. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1749−1752.
(22) Yoshiizumi, K.; Ikeda, S.; Goto, K.; Morita, T.; Nishimura, N.; Sukamoto, T.; Yoshino, K. Synthesis and Structure−Activity Relationships of Novel Phenylcyanoguanidine Derivatives As Potassium Channel Openers. *Chem. Pharm. Bull. (Tokyo)* **1996**, *44*, 2042−2050.
(23) Manley, P. W.; Quast, U. Structure−Activity Studies of Potassium Channel Opening in Pinacidil- Type Cyanoguanidines, Nitroethenediamines, Thioureas, and Ureas. *J. Med. Chem.* **1992**, *35*, 2327−2340.
(24) Atwal, K. S.; McCullough, J. R.; Hedberg, A.; Conder, M. L.; Ahmed, S. Z.; Cucinotta, G.; Normandin, D. E. The Discovery of a Novel Calcium Channel Blocker Related to the Structure of Potassium Channel Opener Cromakalim. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1475−1478.
(25) Yoshiizumi, K.; Ikeda, S.; Nishimura, N.; Yoshino, K. Synthesis and Structure−Activity Relationships of Thienylcyanoguanidine Derivatives as Potassium Channel Openers. *Chem. Pharm. Bull. (Tokyo)* **1997**, *45*, 2005−2010.
(26) Schou, C.; Ottosen, E. R.; Petersen, H. J.; Bjorkling, F.; Latini, S.; Hjarnaa, P. V.; Bramm, E.; Binderup, L. Novel Cyanoguanidines With Potent Oral Antitumour Activity. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 3095−3100.
(27) Lebrun, P.; Arkhammar, P.; Antoine, M. H.; Nguyen, Q. A.; Hansen, J. B.; Pirotte, B. A Potent Diazoxide Analogue Activating ATP−Sensitive K+ Channels and Inhibiting Insulin Release. *Diabetologia* **2000**, *43*, 723−732.
(28) Khan, S. A.; Higdon, N. R.; Hester, J. B.; Meisheri, K. D. Pharmacological Characterization of Novel Cyanoguanidines As Vascular KATP Channel Blockers. *J Pharmacol. Exp. Ther.* **1997**, *283*, 1207−1213.
(29) Lebrun, P.; Antoine, M. H.; Herchuelz, A. K+ Channel Openers and Insulin Release. *Life Sci.* **1992**, *51*, 795−806.
(30) Lebrun, P.; Antoine, M. H.; Ouedraogo, R.; Kane, C.; Dunne, M.; Hermann, M.; Herchuelz, A.; Masereel, B.; Delarge, J.; de Tullio, P.; Pirotte, B. Activation of ATP-Dependent K+ Channels and Inhibition of Insulin Release: Effect of BPDZ 62. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 156−162.
(31) Lebrun, P.; Malaisse, W. J.; Herchuelz, A. Evidence for Two Distinct Modalities of CA2+ Influx into Pancreatic B Cell. *Am. J. Physiol.* **1982**, *242*, E59−E66.
(32) Ashcroft, F. M.; Rorsman, P. Electrophysiology of the Pancreatic Beta-Cell. *Prog. Biophys. Mol. Biol.* **1989**, *54*, 87−143.
(33) Malaisse, W. J.; Lebrun, P. Mechanisms of Sulfonylurea-Induced Insulin Release. *Diabetes Care* **1990**, *13 Suppl 3*, 9−17.
(34) Antoine, M. H.; Hermann, M.; Herchuelz, A.; Lebrun, P. Ionic and Secretory Response of Pancreatic Islet Cells to Minoxidil Sulfate. *J. Pharmacol. Exp. Ther.* **1991**, *258*, 286−291.
(35) Tagmose, T. M.; Mogensen, J. P.; Agerholm, P. C.; Arkhammar, P. O. G.; Wahl, P.; Worsaae, A.; Hansen, J. B. Phenylcyanoguanidines As Inhibitors of Glucose-Induced Insulin Secretion From Beta Cells. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1749−1752.
(36) Dabrowski, M.; Larsen, T.; Ashcroft, F. M.; Hansen, J. B.; Wahl, P. Potent and Selective Activation of the Pancreatic Beta-Cell Type K-ATP Channel by Two Novel Diazoxide Analogues. *Diabetologia* **2003**, *46*, 1375−1382.
(37) Leclercq-Meyer, V.; Marchand, J.; Woussen-Colle, M. C.; Giroix, M. H.; Malaisse, W. J. Multiple Effects of Leucine on Glucagon, Insulin, and Somatostatin Secretion From the Perfused Rat Pancreas. *Endocrinology* **1985**, *116*, 1168−1174.

JM031018Y