Synthesis of and Radioligand Binding Studies with a Tritiated Pinacidil Analogue: Receptor Interactions of Structurally Different Classes of Potassium Channel Openers and Blockers

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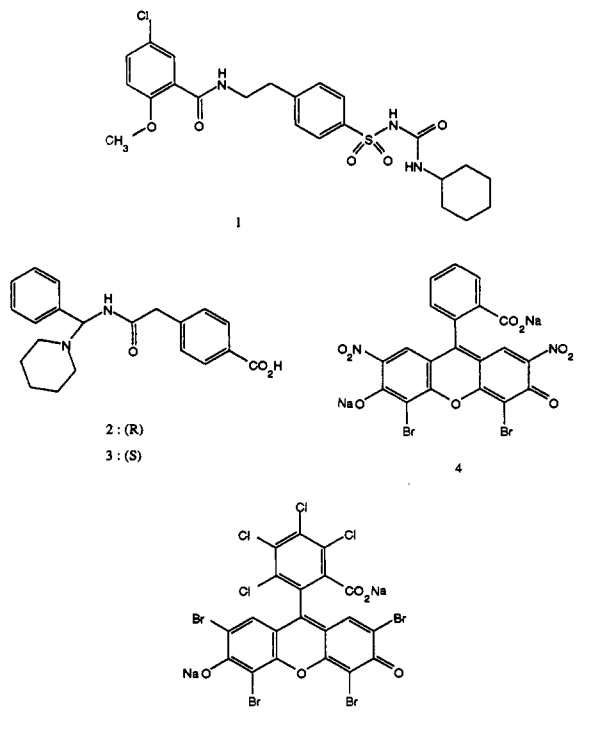
The synthesis of *N*-cyano-*N'*-[1,1-dimethyl-[2,2,3,3-^{3}H]propyl]-*N''*-(3-pyridinyl)guanidine, [^{3}H]-**15**, is described. The utility of this tritiated radioligand in characterizing the interactions of potassium channel openers and blockers with their receptors is demonstrated. Potassium channel openers of the pinacidil, cromakalim, aprikalim, diazoxide, and minoxidil types, as well as K_{ATP} channel blockers of the glibenclamide and eosine types, are all capable of displacing [^{3}H]-**15** from its receptor. The results indicate that all of these compounds interact with the same target protein, but that several different allosterically coupled receptor binding sites are probably involved. The highly significant correlation between the ability of the structurally diverse potassium channel openers to inhibit [^{3}H]-**15** binding and to relax vascular smooth muscle is consistent with their receptor binding sites being closely associated with the potassium channel protein which is the functional target of this class of drugs.

Plasmalemmal potassium channels play a fundamental role in regulating transmembrane electrical potential, and pharmacological modulation of these ion channels by exogenous mediators is proving beneficial in a wide range of therapeutic areas.[1-3] Potassium channels can be classified according to their gating mechanisms into two large groups: voltage-operated channels (K_V) and ligand-gated channels. K_V are usually activated by depolarizing voltages and include the delayed rectifier type, which control cardiac action potential duration and are blocked by class III antiarrhythmic agents.[4] Ligand-gated channels include G-protein linked channels, activated via receptor coupling, as for example in atrium where potassium channels are opened through acetylcholine coupling to associated M_2 muscarinic receptors,[5] and calcium-dependent potassium channels (K_{Ca}), which although regulated by intracellular calcium also show a strong voltage dependence.[6]

Another important class of ligand-gated potassium channels are adenosine 5'-triphosphate sensitive potassium channels (K_{ATP}).[7-9] K_{ATP} are closed by intracellular ATP (ATP^{4-}), and this inhibition is modulated by other nucleotides, such as MgADP, so that these channels couple cellular excitability to the metabolic state of the cell. K_{ATP} are well characterized in pancreatic β-cells, where elevated plasma glucose levels cause an increase in intracellular ATP resulting in closure of K_{ATP} channels, depolarization of the cell, Ca^{2+} entry, and subsequent insulin secretion. Similar mechanisms involving K_{ATP} govern neurotransmitter release in several regions of the brain.[10,11] K_{ATP} are found in all muscle types (heart, skeletal, and smooth muscle) where they are normally closed but are opened under ischaemic conditions, when intracellular concentrations of ATP fall.[8,9] In most tissues K_{ATP} are blocked by hypoglycaemic sulfonylureas, such as glibenclamide (glyburide; 1, Chart I) or structurally related compounds used in the treatment of type II diabetes, where they decrease the potassium permeability of pancreatic β-cells.[7-9] Binding studies with radiolabeled 1 have shown the presence of receptors for this ligand in insulin-secreting tumor cells and β-cell lines,[12,13] cardiiac ventricular cells,[14,15] intestinal smooth muscle,[15,16] and the brain.[12,15] However, despite intense efforts to purify the glibenclamide receptor from brain and β-cell lines, the evidence that this large (140 kD), high-affinity receptor is an integral part of K_{ATP} is still indirect.[17,18] Therefore, the relevance of binding studies with radiolabeled 1 for the characterization of all K_{ATP} remains unclear.

Chart I. Two Structurally Different Types of Potassium Channel Blockers[a]



[a] Glibenclamide-type, represented by glibenclamide (1) and the enantiomers of AZ-DF 265 (2 and 3), and fluorescein-type, represented by eosin (4) and phloxin B (5).

The potassium channel openers constitute a structurally diverse class of drugs (Chart II), which are believed to act

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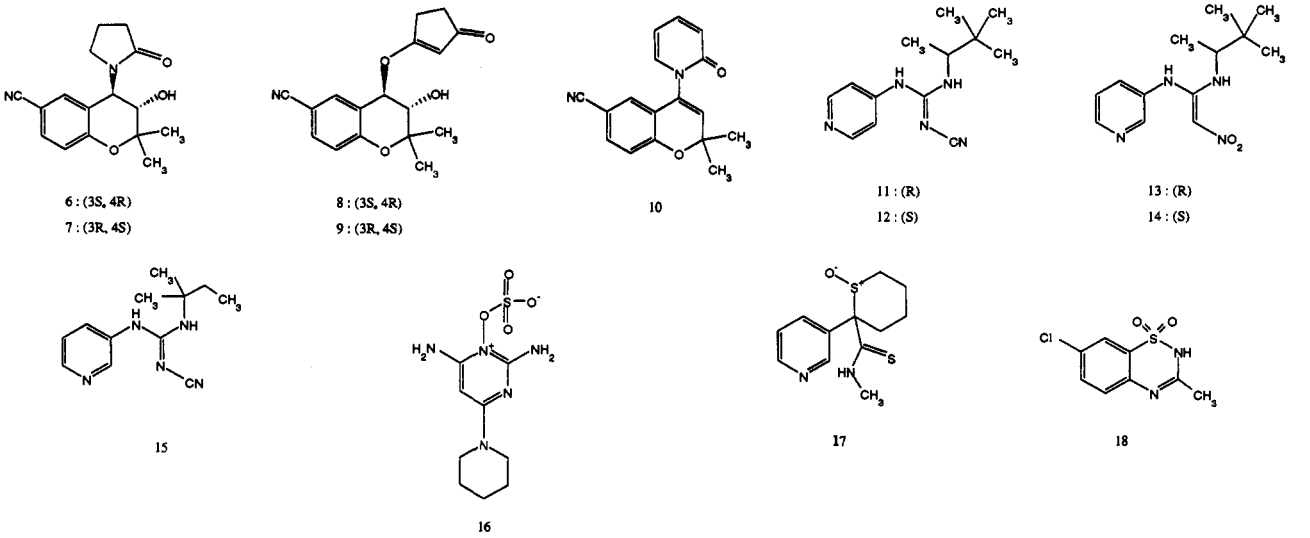
Chart II. Five Structurally Different Types of Potassium Channel Openers[a]



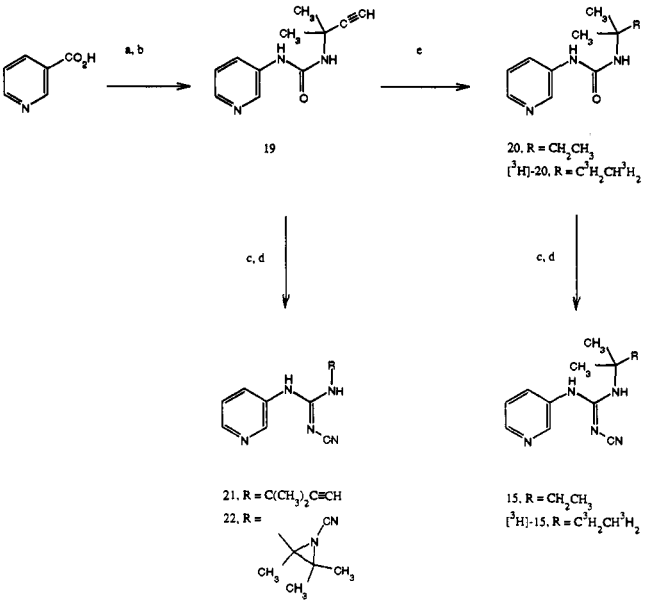
[a] Cromakalim-type, represented by the enantiomers of cromakalim (6 and 7) and PCO 400 (8 and 9) and by bimakalim (10); pinacidil-type, represented by the enantiomers of pinacidil (11 and 12) and the nitroethenediamines (13 and 14) and by P1075 (15); minoxidil-type, represented by minoxidil sulfate (16); aprikalim-type, represented by the racemate of aprikalim, RP49356 (17); and diazoxide-type, represented by diazoxide (18).

principally by activating potassium channels in vascular and airway smooth muscle cells, thereby exerting a relaxatory effect, through which they have potential therapeutic utility as blood pressure lowering and bronchodilatatory agents.[1-3] The *in vitro* stimulation of K^+ efflux and vasorelaxation, as well as the *in vivo* activities of these compounds, is antagonized by K_{ATP} blockers, such as glibenclamide, suggesting that their site to action may be K_{ATP}.[19] Further evidence for this mode of action comes from whole cell current measurements in vascular smooth muscle, which have clearly shown that potassium channel openers open an ATP-modulated channel.[20,21] However, on the basis of studies at the single-channel level, K_{ATP} having significantly different biophysical properties have been proposed to be the target of cromakalim (the racemate of 6) or pinacidil (racemate of 11).[22,23]

In summary, the exact nature of the K_{ATP} opened by these compounds remains unclear and consequently it is unknown whether or not all of the structurally heterogeneous potassium channel openers bind to the same channel. Clearly, the availability of a suitable radioligand would greatly facilitate the identification of the target potassium channels, and a binding assay could establish whether or not structurally diverse potassium channel activators would compete for the same binding sites. Furthermore, it would also be possible to ascertain whether or not individual potassium channel blockers exert their effects by directly inhibiting potassium channel opener receptor binding or through an indirect mechanism.

From our *in vitro* structure–activity studies on the pinacidil type of potassium channel activators,[24] the cyanoguanidine **15** (P-1075[25]) appeared to fulfill the criteria of potency and hydrophilicity suitable for a radioligand. In this publication we report the synthesis of the tritiated radioligand [^{3}H]-**15**. Using the binding assay established for this radiolabel,[26] the effects of representative potassium channel openers and blockers (Charts I and II) on [^{3}H]-**15** binding were examined. Results from these experiments suggest that the pinacidil, cromakalim, aprikalim, diazoxide, and minoxidil types of potassium channel activator, as well as the glibenclamide-type potassium channel blockers, bind to discrete, allosterically coupled, receptor binding sites located on the same K_{ATP} protein complex.

Scheme I.[a] Synthesis of **15** and [^{3}H]-**15** from Nicotinic Acid



[a] Reagents: (a) $(PhO)_2PON_3$, Et_3N, C_6H_6; (b) $HC{\equiv}CCMe_2NH_2$; (c) PPh_3, CCl_4, Et_3N; (d) H_2NCN, i-Pr_2NEt; (e) Pd, H_2, EtOAc.

Synthesis

The most expedient synthesis of the desired tritiated cyanoguanidine would involve tritiation of the acetylenic precursor **21**. However, attempted dehydration of the readily available acetylenic urea **19** followed by *in situ* addition of cyanamide to the intermediate carbodiimide, under the usual conditions,[23] failed to give the desired cyanoguanidine **21**, with the cyanoaziridine **22** being the only isolable product (Scheme I). Consequently, we decided to tritiate **19**, prior to conversion to the cyanoguanidine (Scheme I). Thus treatment of nicotinic acid with diphenyl phosphorazidate afforded the acyl azide, Curtius rearrangement of which, followed by *in situ* reaction of the resulting isocyanate with the required

butynamine, gave the acetylenic area **19** in 74% yield. Catalytic hydrogenation of **19** afforded **20**, which on treatment with carbon tetrachloride and triphenylphosphine in the presence of triethylamine gave the intermediate carbodiimide, which was reacted with cyanamide to give the corresponding cyanoguanidine **15**. Similarly, for the radiolabeled material, tritiation of **19** afforded [^{3}H]-**20**, which was subsequently converted to [^{3}H]-**15** with a radiochemical purity of >98%. The UV spectrum (MeOH) of [^{3}H]-**15** was indistinguishable from that of unlabeled material (λ_{max} 238 nm, ϵ 16 377), and the specific activity was calculated to be 86.7 Ci/mmol. The compound was stored on EtOH at −80 °C and appears to be quite stable in radiolabeled form since HPLC analysis after 2.5 years showed only minor impurities and <15% decrease in radiochemical purity.

Biological Evaluation

Binding of [^{3}H]-**15** was evaluated as described.[26] Nonspecific binding (determined by incubating the tissue in the presence of 1 μM unlabeled ligand) was in the range of 35–40% of total binding. Endothelium-denuded intact rings of rat aorta (2–3 mm) were incubated, together with [^{3}H]-**15** (0.3 nM) and the competing drug under study, at 37 °C for 90 min in physiological medium. The rings were washed with ice-cold medium for 1 min, blotted free from medium, solubilized with Lumasolv (0.5 mL; Facola, Basel), supplemented with HCl (0.5 mL of 0.1 M), and counted in Optifluor (16 mL; Packard, Zurich) by liquid scintillation spectroscopy.

The functional, vasorelaxant activity of the potassium channel openers was evaluated by means of their effect on spontaneous myogenic activity in rat portal veins, as previously reported,[24] with the molar concentrations required to inhibit the contractile activity by 50%, expressed as pD_2 ($-\log D_2$).

Data are presented as means ±SEM from three to five experiments. Concentration–response curves were analyzed by performing unweighted least squares fits of the data to the Hill equation, and errors were calculated by propagation of errors using linear approximations and neglecting covariances.

Results and Discussion

The binding of [^{3}H]-**15** to rat aorta rings has been shown to satisfy the generally accepted requirements for receptor binding.[26] Binding is saturable (maximum binding capacity, B_{max} = 21 ± 3 fmol/mg of tissue wet weight), of high affinity (K_D = 6 ± 1 nM), and reversible (half-life of complex = 19 min).

The inhibition of specific [^{3}H]-**15** binding data obtained in this study is illustrated for the enantiomers of cromakalim, **6** (levcromakalim), and its distomer **7**, **10** (bimakalim), and the nitroethenediamines **13** and **14** in Figure 1. All of the compounds examined completely inhibited specific binding in a concentration-dependent manner (the extent of inhibition being approximately 65% of total binding), with the exception of minoxidil sulfate **16** which inhibited by 86%. The Hill coefficients were close to unity. The midpoint of the inhibition curves, expressed as pK_i ($-\log K_i$), of the compounds tested are given in Table I.

From the data in Table I, it can be seen that all of the potassium channel activators inhibit specific binding of [^{3}H]-**15** to its receptor in rat aorta irrespective of whether

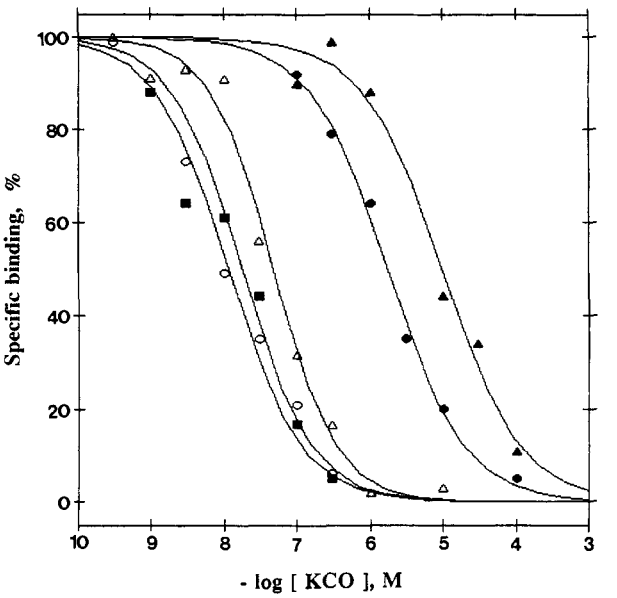


Figure 1. Inhibition of specific [^{3}H]-**15** binding in endothelium denuded rings of rat aorta by levcromakalim (Δ, **6**) and its distomer (▲, **7**), bimakalim (■, **8**), and the enantiomeric nitroethenediamines (●, **13** and ○, **14**). The data are the means ±SEM from three to five experiments. The fit of the data to the Hill equation yielded the pK_i values listed in Table I; the slopes of the curves (Hill coefficients) are in the range 0.81–1.02. Nonspecific binding, determined in the presence of 1 μM **15**, was 35–40% of total binding.

Table I. Receptor Binding and Vasorelaxant Properties of Potassium Channel Activators and Potassium Channel Blockers

compd[a]	function	optical rotation[b] $[\alpha]^{20}_D$	binding pK_i[c]	vasorelaxant effects pD_2[d]
6	opener	−54.7[e]	7.33 ± 0.06	7.89 ± 0.03
7	opener	+53.4[e]	5.02 ± 0.09	5.66 ± 0.08
8	opener	−129.1	7.21 ± 0.06	7.85 ± 0.03
9	opener	+129.9	5.06 ± 0.03	5.26 ± 0.06
10	opener	achiral	7.74 ± 0.04	8.20 ± 0.04
11	opener	−147.9	7.66 ± 0.06	7.47 ± 0.01
12	opener	+144.9	6.27 ± 0.16	6.11 ± 0.03
13	opener	−195.7	5.77 ± 0.03	6.00 ± 0.08
14	opener	+195.9	7.96 ± 0.10	7.96 ± 0.04
15	opener	achiral	8.54 ± 0.03	8.75 ± 0.05
16	opener	achiral	7.45 ± 0.10[f]	7.14 ± 0.03
17	opener	racemate	6.75 ± 0.05	6.94 ± 0.04
18	opener	achiral	4.66 ± 0.03	5.50 ± 0.04
1	blocker	achiral	6.36 ± 0.04	
2	blocker	(+)[g]	5.86 ± 0.04	
3	blocker	(−)[h]	5.63 ± 0.05	
4	blocker	achiral	≈4	
5	blocker	achiral	≈3	

[a] For structural formulas see Charts I and II. [b] c = 1.0 (EtOH). [c] Negative logarithm of the inhibition constant, K_i, of [^{3}H]-**15** binding. [d] For potassium channel activators: negative logarithm of the concentration causing a 50% inhibition of spontaneous activity in rat portal vein. [e] c = 0.5 ($CHCl_3$). [f] Displaces only 80% of specific binding. [g] Enantiomeric excess 94.4%. [h] Enantiomeric excess 98.6%.

or not they are structurally related to the cyanoguanidine **15**. The compounds also inhibit spontaneous electrical activity, concentration dependently, in rat portal vein, yielding the pD_2 values listed in Table I. The excellent correlation (Figure 2; correlation coefficient r = 0.959, slope = 0.88 ± 0.08) between these two sets of data, indicates that the activities of the potassium channel openers in the two assays are directly proportional to one another. The ability of potassium channel openers to inhibit spontaneous myogenic activity in rat portal vein has been shown to be directly related to their ability to stimulate $^{86}Rb^+$ efflux from this tissue and hence to activate potassium chan-

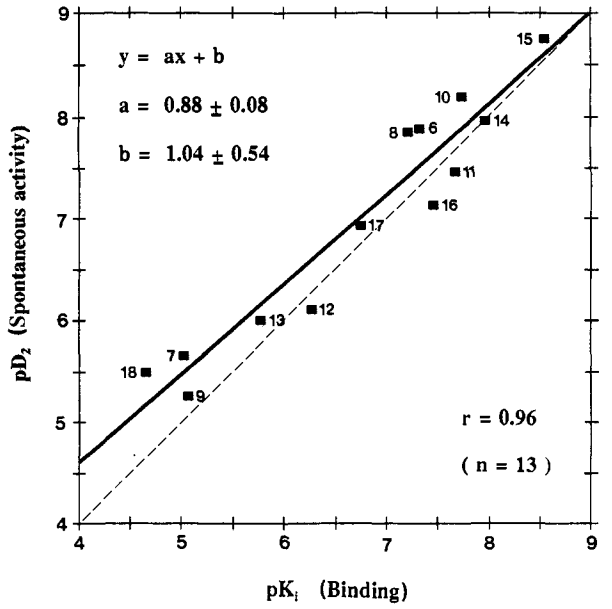


Figure 2. Comparison of the potencies of the potassium channel openers as inhibitors of specific [^{3}H]-15 binding (pK_i) in rat aorta and as inhibitors of rat portal vein spontaneous activity (pD_2). Linear correlation analysis of the data (solid line) gave the following: correlation coefficient, $r = 0.96$; slope 0.88 ± 0.08 ($n = 13$). The broken line is the line of identity, $y = x$.

nels.[24,27] Therefore, it may be concluded that the single class of binding sites labeled by [^{3}H]-15 is associated with a protein which is the receptor target of most of the known classes of potassium channel openers and which is very similar in both rat aorta and rat portal vein. This protein therefore probably constitutes at least a part of the potassium channel (K_{PCO}) which is activated by the potassium channel openers.

The above considerations do not differentiate between the potassium channel openers either interacting with different binding sites which are negatively allosterically coupled to the [^{3}H]-15 binding site, such that when they occupy their receptors the [^{3}H]-15 binding site is masked by a conformational change in the receptor protein, or directly interacting with the [^{3}H]-15 binding site itself. However, whereas very similar concentrations of compounds of the pinacidil type (compounds 11–15) are required to inhibit both radioligand binding and spontaneous myogenic activity (Figure 2), significantly higher concentrations of the benzopyrans 6-8 and 10 and diazoxide 18 are required to inhibit [^{3}H]-15 binding than are required to inhibit spontaneous activity. This is clearly seen in Figure 2, when the positions of the different groups of compounds are considered with respect to the line of identity (broken line): compounds of the pinacidil type lie on or very close to the line and benzopyran type compounds lie above the line; RP49356 (17) is slightly above and minoxidil sulfate (16) significantly below the line, and these latter two classes of compounds require further study to clarify their situation. This observation suggests that the benzopyran and diozoxide type potassium channel openers inhibit binding allosterically, with this inhibition being less efficient than the competitive inhibition exerted by the pinacidil analogues. Theoretically, this hypothesis could be tested by performing [^{3}H]-15 saturation experiments in the presence of the unlabeled inhibitor. However, technical problems preclude this possibility (see the experimental scatter in previously reported saturation experiments[26]). An important consequence of this hypothesis is that attempts to develop a common pharmacophore model for the pinacidil and benzopyran types of potassium channel openers and to improve potency and selectivity through the design of hybrid structures are unlikely to be rewarding.

From the current literature, it is unclear as to whether K_{ATP} blockers, such as glibenclamide 1, antagonize the activities of potassium channel openers by binding to the same receptor site, or through some other mechanism. Thus, whereas diazoxide 18 has been reported as being a modestly effective inhibitor of specific binding of tritiated glibenclamide [^{3}H]-1, in both rat cardiac and cortex membranes, cromakalim, levcromakalim 6, and pinacidil were all found to be ineffective.[15] In another study, (±)-aprikalim, 17, completely displaced [^{3}H]-1 from its receptor in guinea pig ileum smooth muscle, while diazoxide (18), cromakalim, and levcromakalim (6) were without effect.[16] It has also been reported that pinacidil, although ineffective at displacing [^{125}I]-1 from rat brain homogenates and [^{3}H]-1 from both HIT cell and rat cerebral cortex microsomes,[27,28] can displace [^{3}H]-1 in the latter two preparations at high concentrations (>200 μM) in the presence of MgATP. In contrast to these reports, in the present work utilizing rat aorta, specific binding of the pinacidil analogue, [^{3}H]-15, was concentration-dependently inhibited by glibenclamide, as well as by the (*R*)- and (*S*)-enantiomers of AZ-DF 265, 2 and 3, at submicromolar concentrations. In terms of their insulinotropic activity, it is reported that 1 and 3 are approximately equipotent, with 2 being 10 times less active *in vitro* and 15 times less active *in vivo* than 3.[29] However, in their ability to inhibit [^{3}H]-15 binding in rat aorta, the enantiomers of AZ-DF 265 displayed little stereoselectivity, with the more potent (*R*)-enantiomer 2 being approximately 3 times less active than glibenclamide 1. This lack of stereoselectivity in inhibiting [^{3}H]-15 binding is in marked contrast to the high degree of stereoselectivity observed for the potassium channel openers (Table I). Therefore, it appears that there are major differences between the receptor interaction of the AZ-DF 265 enantiomers with potassium channels in rat aorta and in insulin-secreting cells. However, these results are consistent with the hypoglycaemic K_{ATP} blockers antagonizing the activities of the potassium channel openers by binding to a receptor which is negatively allosterically coupled to the [^{3}H]-15 binding site located on a potassium channel in rat aorta.[25] Consequently, the reported inability of several types of potassium channel openers to inhibit radiolabeled glibenclamide binding in several tissues could arise from the allosteric interactions from the potassium channel opener sites to the glibenclamide site being weak, whereas the converse inhibitory allosteric coupling from the glibenclamide site to the [^{3}H]-15 site is much stronger. The nature and extent of this allosteric coupling could also vary from tissue to tissue. Good precedent for such asymmetry in allosteric coupling is found in the GABA–benzodiazepine receptor system.[30]

In order for potassium channel openers such as cromakalim, diazoxide, pinacidil, or RP 49356 to active K_{ATP} in β-cells or vascular smooth muscle cells, intracellular ATP must be present.[21,31,32] Also it is becoming apparent that the modulation of K_{ATP} channels by nucleotides involves two distinct nucleotide-binding sites on the intracellular face of the channel.[7-9] These observations may be explained by a model in which occupation of an

activatory, nucleotide binding site (A-site) allows activation of the channel, whereas occupation of an inhibitory, nucleotide binding site (I-site), or occupation of both A- and I-sites, inhibits the channel. Consistent with this model, in insulinoma cells eosin (4) and phloxine B (5), established ligands for the ATP-binding site in (Na,K)-ATPases,[33] can inhibit active K_{ATP} as well as reactivate K_{ATP} which have been subjected to inactivation ("run-down") through the absence of cytoplasmic ATP.[34] According to this model gibenclamide exerts its inhibitory effect by occupying a binding site which is coupled in a negative allosteric manner to the A-site.[34,35] In order to provide information regarding those A- and I-nucleotide binding sites and the [^{3}H]-15 binding site, the effects of eosin and phloxine B on [^{3}H]-15 binding were investigated. Both compounds inhibited binding (Table I) in a concentration-dependent manner, with potencies of a similar order of magnitude as those required to inhibit $^{86}Rb^+$ efflux from insulinoma cells.[34] This data therefore provides further evidence suggesting that the channel protein, targeted by the potassium channel openers in vascular smooth muscle, has an ATP-binding domain. Since intracellular ATP also appears to be a prerequisite K_{ATP} activation by potassium channel openers in smooth muscle,[21] the inhibition of binding by eosin and phloxin B is probably the result of a negative allosteric interaction between an inhibitory nucleotide site and the [^{3}H]-15 binding site. Thus it is reasonable to postulate that potassium channel openers of the pinacidil type activate K_{ATP} in smooth muscle by interfering with nucleotide binding at the I-site, in a manner similar to that in the multiple ligand binding model proposed for K_{ATP} regulation in cardiac cells.[36] The testing of this hypothesis requires radioligand binding in membranes, and the development of such an assay is currently being investigated.

Finally, it is interesting to speculate that minoxodil sulfate, 16, which shows atypical behavior as compared to the other non-pinacidil-type potassium channel openers in its potency in displacing [^{3}H]-15 from its receptor and has some structural and electrostatic potential features similar to those of ATP, could exert its effects on K_{ATP} by mimicking ATP. Then, on the basis of the above model, 16 would then bind to the A-site as an ATP agonist or, alternatively, to the I-site as an antagonist.

Conclusion

This paper describes the first synthesis of a radioligand capable of labeling receptor binding sites which are the functional target of potassium channel openers in smooth muscle. The results suggest that different structural types of potassium channel opener probably interact with different receptor binding sites which are allosterically coupled. Since glibenclamide 1 inhibits [^{3}H]-15 binding in rat aorta, but potassium channel openers of the pinacidil-type do not inhibit [^{3}H]-1 binding in various tissues, it is postulated that the allosteric interactions from the potassium channel opener sites to the glibenclamide site are weak, whereas the converse inhibitory allosteric coupling from the glibenclamide site to the [^{3}H]-15 site is much stronger. Since the nature and extent of this allosteric coupling could vary from tissue to tissue, potassium channel openers should not be characterized solely on the basis of their antagonism by glibenclamide.

Experimental Section

Chemistry. General Methods. Reagents, starting materials, and solvents were purchased from common commercial suppliers and were used as received or distilled from an appropriate drying agent. Evaporations were carried out on a rotary evaporator at bath temperatures <50 °C and under an appropriate vacuum. Medium-pressure column chromatography was performed using silica gel (E. Merck, Grade 60, particle size 0.040-0.063 mm, 230-400-mesh ASTM) with the solvent system indicated. Melting points were determined with a Buchi-Tottoli apparatus in open capillary tubes and are uncorrected. Proton NMR spectra were recorded at ambient temperature with a Brucker Spektrospin WH 360 apparatus (360 MHz) using Me_4Si as an internal standard; chemical shifts are reported in δ values (ppm) relative to internal Me_4Si. Where elemental analyses are indicated by the symbols of the elements, they were performed by the analytical department at Sandoz and results obtained were within ±0.4% of the theoretical values.

Compound 6-16 were prepared by methods described in the literature[24,35-38] and were characterized by proton NMR and elemental analysis data which were fully in accordance with the expected structures; the enantiomeric excesses of chiral compounds were >99%, as established by proton NMR in the presence of a chiral shift reagent, and optical rotations are given in Table I. Compounds 1, 4, and 5 were purchased from Sigma, and compounds 2 and 3 (Dr. Karl Thomae, GmbH, Biberach an der Riss), 17 (Rhône Poulenc Rorer, Paris), and 18 (Essex Pharma, München) were generously supplied by the companies indicated.

***N*-(1,1-Dimethyl-2-propynyl)-*N'*-(3-pyridinyl)urea (19).** Diphenyl phosphorazidate (12.5 mL, 58 mmol) was added to a stirred solution of nicotinic acid (6.16 g, 50 mmol) and triethylamine (7.6 mL, 57 mmol) in toluene (100 mL) under argon. The solution was stirred at 25 °C for 3 h, treated with 2-methyl-3-butyn-2-amine (6.0 mL, 57 mmol), and heated at 60 °C for 16 h. The solvent was evaporated under reduced pressure, treated with saturated aqueous $NaHCO_3$ (100 mL), and extracted with CH_2Cl_2 (3 × 100 mL). The combined extracts were dried (Na_2SO_4), and the solvent was evaporated to give the crude product which was purified by chromatography (silica gel, eluent 0.5% NH_4OH, 4.5% EtOH in CH_2Cl_2) and recrystallized from CH_2Cl_2–Et_2O to give 19 (7.5 g, 74%) as a colorless crystalline solid: mp 123–124 °C; ^{1}H NMR ($CDCl_3$) δ 1.64 (s, 6, CMe_2), 2.43 (s, 1, ≡CH), 5.63 (br s, 1, NH), 7.22 (dd, $J_{5,6}$ = 4.7 Hz, $J_{4,5}$ = 8.4 Hz, 1, 5-PyrH), 7.88 (br s, 1, NH), 8.09 (dq, $J_{4,6}$ = 1.4 Hz, $J_{2,4}$ = 2.6 Hz, $J_{4,5}$ = 8.4 Hz, 1, 4-PyrH), 8.21 (dd, $J_{4,6}$ = 1.4 Hz, $J_{5,6}$ = 4.7 Hz, 1, 6-PyrH), and 8.32 (d, $J_{2,4}$ = 2.6 Hz, 1, 2-PyrH). Anal. ($C_{11}H_{13}N_3O$) C, H, N.

***N*-(1,1-Dimethylpropyl)-*N'*-(3-pyridinyl)urea (20).** A solution of 19 (1.02 g, 5.0 mmol) in EtOAc (150 mL) was hydrogenated at room temperature and atmospheric pressure over 10% palladium on carbon (0.10 g). Hydrogen uptake was complete (240 mL) after 10 min. The catalyst was removed by filtration, and the solvent was evaporated to give a residue which recrystallized from Et_2O to give 20 (0.85 g, 82%) as a colorless crystalline solid: mp 109–110 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, J = 7.5 Hz, 3, CH_2Me), 1.31 (s, 6, CMe_2), 1.71 (q, J = 7.5 Hz, 2, CH_2Me), 5.17 (br s, 1 NH), 7.20 (dd, $J_{5,6}$ = 4.7 Hz, $J_{4,5}$ = 8.4 Hz, 1, 5-PyrH), 7.64 (br s, 1, NH), 8.05 (dq, $J_{4,6}$ = 1.4 Hz, $J_{2,4}$ = 2.5 Hz, $J_{4,5}$ = 8.4 Hz, 1, 4-PyrH), 8.18 (dd, $J_{4,6}$ = 1.4 Hz, $J_{5,6}$ = 4.7 Hz, 1, 6-PyrH), and 8.26 (d, $J_{2,4}$ = 2.5 Hz, 1, 2-PyrH). Anal. ($C_{11}H_{17}N_3O$).

***N*-[1,1-Dimethyl-[2,2,3,3-^{3}H]propyl]-*N'*-(3-pyridinyl)urea ([^{3}H]-20).** A 1.0-mL hydrogenation flask containing 19 (6.5 mg, 0.032 mmol) and 10% palladium on carbon (9 mg) in redistilled EtOAc (0.5 mL) was degassed, cooled to 193 K, and evacuated. Tritium gas (20 Ci) was then introduced, and reduction was allowed to proceed at room temperature for 2 h at 0.5 atm followed by an additional 1 h at 1.0 atm. The catalyst was removed by millipore filtration, and the solvent was evaporated to give the product which was freed from labile tritium by repeated addition of MeOH (5 mL) followed by evaporation to dryness. For storage, the product was dissolved in MeOH (135 mL) to give a solution having a specific activity of 14.95 mCi/mL.

N-Cyano-N'-[1,1-dimethyl-[2,2,3,3-^{3}H]propyl]-N''-(3-Pyridinyl)guanidine ([^{3}H]-15). A 1.0-mL glass ampoule containing [^{3}H]-20 (3.5 mg, 0.017 mmol; total radioactivity 942 mCi), triphenylphosphine (20 mg, 0.076 mmol), carbon tetrachloride (20 mg, 0.13 mmol), triethylamine (20 mg, 0.20 mmol), and dichloromethane (50 μL) was sealed under argon and heated at 35 °C for 16 h. The mixture was evaporated to dryness to give the crude carbodiimide, which was treated with dry cyanamide (30 mg, 0.71 mmol), *N,N*-diisopropylethylamine (20 μL), and CH_2Cl_2 (50 μL) and heated at 35 °C for 4 h. The mixture was evaporated to dryness, and the residue was treated with hydrochloric acid (2 mL of 1.0 M) and extracted with EtOAc (3 × 3 mL). The combined extracts were washed with brine (2 × 2 mL), and the combined aqueous phases were neutralized with saturated aqueous $NaHCO_3$ and extracted with EtOAc (2 × 3 mL). The combined extracts were then evaporated to give the crude product, which was purified by column chromatography on silica gel (1 g, 230–400-mesh ASTM), eluent 25% aqueous NH_3–EtOH–CH_2Cl_2 (0.5:2:98) to afford ca. 2.0 mg of [^{3}H]-15 (57%), specific activity 86.7 Ci/mmol, and a 99.3% HPLC purity.

N-Cyano-N'-(N-cyano-2,3,3-trimethylaziridin-2-yl)-N''-(3-pyridinyl)guanidine (22). A mixture of 19 (3.80 g, 18.7 mmol), triphenylphosphine (7.00 g, 26.7 mmol), triethylamine (3.0 mL, 22 mmol), and CCl_4 (3.0 mL, 31 mmol) in dry CH_2Cl_2 (60 mL) was heated under reflux in an argon atmosphere for 8 h. The mixture was evaporated to dryness, and the residue was triturated with dry ether (3 × 100 mL). The combined extracts were dried (K_2CO_3), and filtered, and the solvent was evaporated to give a residue which was treated with cyanamide (1.20 g, 28.6 mmol) and *N*-ethyldiisopropylamine (1.2 mL, 7.2 mmol) in dry CH_2Cl_2 (10 mL), and heated at 35 °C for 16 h. The solvent was evaporated, and the residue was purified by column chromatography (silica gel, 0.5% NH_4OH, 4.5% EtOH in CH_2Cl_2) and recrystallized from EtOH–EtOAc to give 22 (1.45 g, 29%) as a colorless crystalline solid: mp 192–193 °C; ^{1}H NMR (DMSO-d_6) δ 1.32 (s, 3, Me), 1.35 (s, 3, Me), 1.41 (s, 3, Me), 3.37 (br s, 1, NH), 7.55 (dd, $J_{5,6}$ = 4.8 Hz, $J_{4,5}$ = 8.1 Hz, 1, 5-PyrH), 7.82 (dq, $J_{4,6}$ = 1.6 Hz, $J_{2,4}$ = 2.5 Hz, $J_{4,5}$ = 8.1 Hz, 1, 4-PyrH), 8.20 (br s, 1, NH), 8.57 (d, $J_{2,4}$ = 2.5 Hz, 1, 2-PyrH), 8.62 (dd, $J_{4,6}$ = 1.6 Hz, $J_{5,6}$ = 4.8 Hz, 1, 6-PyrH) and 9.60 (br s, 1, NH); ^{13}C NMR (DMSO-d_6) δ 18.03 (C*Me*CH_3), 20.88 (C*Me*CH_3), 25.02 (CH_3), 63.29 (*C*Me_2), 81.85 (*C*Me), 113.57 (CN), 116.76 (CN), 123.86 (5-Pyr), 130.61 (3-Pyr), 137.33 (4-Pyr), 149.15 (2-Pyr), 150.40 (6-Pyr) and 160.47 (NN*C*=NCN). Anal. ($C_{13}H_{15}N_7$). The structure was confirmed by HCCORR NMR.

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