Binding of ATP-Sensitive Potassium Channel (K_{ATP}) Openers to Cardiac Membranes: Correlation of Binding Affinities with Cardioprotective and Smooth Muscle Relaxing Potencies

Karnail S. Atwal,* Gary J. Grover, Nicholas J. Lodge, Diane E. Normandin, Sarah C. Traeger, Paul G. Sleph, Robert B. Cohen, Catherine C. Bryson, and Keneth E. J. Dickinson

Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, New Jersey 08543-4000

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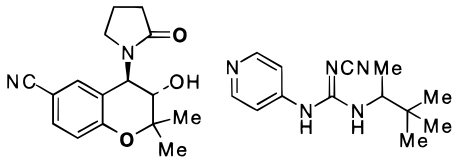
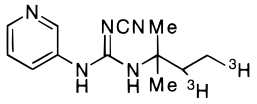
K_{ATP} openers (e.g., cromakalim (**1**), pinacidil (**2**)) are an important class of compounds with potential clinical indications for diseases such as hypertension, asthma, hair growth, ischemia, and urinary incontinence.[1] The major drawback of the first generation agents (e.g., **1**, **2**) is their lack of tissue selectivity which limits clinical utility.[2] The search for tissue selective agents has been hampered by the lack of high-volume screening methods, notably those associated with radioligand binding. Although binding sites for the pinacidil related K_{ATP} opener [^{3}H]P-1075 (**3**) have been reported in intact tissues[3] and cells,[4] efforts to identify binding sites in membrane preparation which are usually amenable to high volume screening have not been successful. We have recently reported that **3** binds to skeletal muscle membranes with high affinity.[5] In this communication we report for the first time the identification of binding sites for **3** in canine myocardial membranes and the correlation of binding affinities to cardioprotective and vasorelaxation potencies for a series of K_{ATP} openers.



Cromakalim (**1**)

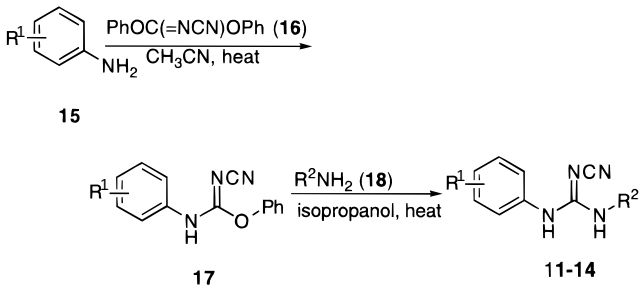
Pinacidil (**2**)



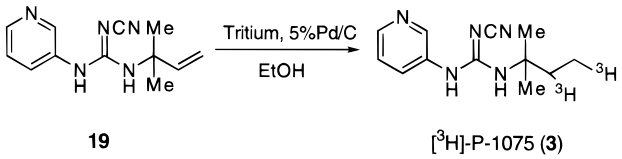
P-1075 (**3**)

Most of the analogues (**4**−**10**) reported in this paper have been described previously.[6] The remaining compounds can be prepared by treatment of **15** with diphenyl cyanocarbonimidate (**16**) to give the intermediate **17** which is converted to the desired products (**11**−**14**) by treatment with amines (**18**) (Scheme 1). Details of this method are described.[7] Physical properties of new analogues are summarized in Table 1. The radioligand (**3**) was prepared from the corresponding olefin **19** by catalytic tritiation (Scheme 2). Compound **19** was generated from 3-aminopyridine and 2,2-dimethylallylamine as described in Scheme 1. The specific activity

Scheme 1



Scheme 2



of **3** was 51−61 Ci/mmol. The K_{ATP} opener pinacidil (**2**) and compounds **4**, **9**, and **10** employed in these studies were all racemates.

As recently reported, the key discovery for the identification of binding sites for **3** in skeletal muscle membranes was the presence of nucleotides (e.g., MgATP) in binding medium.[5] Having established the protocol for the observation of consistent binding in skeletal muscle membranes, we carried out experiments to identify binding sites for **3** in canine cardiac membranes. Our initial studies indicated the existence of specific binding in canine and rat myocardial membranes with similar pharmacological characteristics. For example, **3** has a K_i value of 27 ± 5 and 30 ± 3 nM in canine and rat heart myocardial membranes, respectively. The K_{ATP} blocker glyburide also has similar K_i values in the rat (290 ± 100 nM) and dog (130 ± 20 nM) cardiac membranes. To evaluate a large number of analogues for binding affinities, we employed canine cardiac membranes due to higher supply of the tissue.[8] Binding assays included 240 mM sucrose, 3 mM sodium orthovanadate, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, in a total volume of 1 mL.[9] Displacement studies were conducted by incubation of **3** (14 nM) and inhibitors with membranes (500−700 μg) for 60 min. Nonspecific binding was defined with 10 μM nontritiated **3**. The nucleotide requirements for observation of binding were similar to those described in skeletal muscle membranes.[5] K_i values for various compounds were calculated from IC_{50} values using the equation of Cheng and Prusoff.[10]

The binding to cardiac membranes was saturable, reversible and following addition of nontritiated **3**, the radioligand dissociated from membranes with first-order kinetics ($t_{1/2}$ = 21 min). The K_d value calculated as the ratio of the dissociation and association rate constants was 39 nM. At 14 nM **3**, specific binding represented 87% of total binding. Saturation analysis indicated a single homogeneous population of high-affinity binding sites with a K_d value of 28 ± 5 nM and B_{max} value of 73 ± 8 fmol/mg of protein (n = 4). The binding was maximal at pH 8−9, and it was sensitive to proteolysis

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Table 1. Physical Properties of Compounds **11**−**14** and **19**

compd	mol formula	appearence	mp (°C)[a]	microanalysis
11	$C_{18}H_{27}N_5O_2S$	colorless solid	173−174 (A)	C, H, N, S
12	$C_{18}H_{27}N_5O_2S$	colorless solid	209−210 (A)	C, H, N, S
13	$C_{13}H_{16}N_4I\cdot 0.17H_2O$	colorless solid	152−153 (B)	C, H, N
14[b]	$C_{13}H_{16}N_7I$	off white foam		
19	$C_{12}H_{15}N_5\cdot 0.2H_2O\cdot 1.2$ethyl ether	colorless solid	168−170 (C)	C, H, N

[a] Solvent for crystallization: A, trituration with isopropyl ether; B, hexanes−CH_2Cl_2; C, trituration with ethyl ether. [b] Also reported in ref 15.

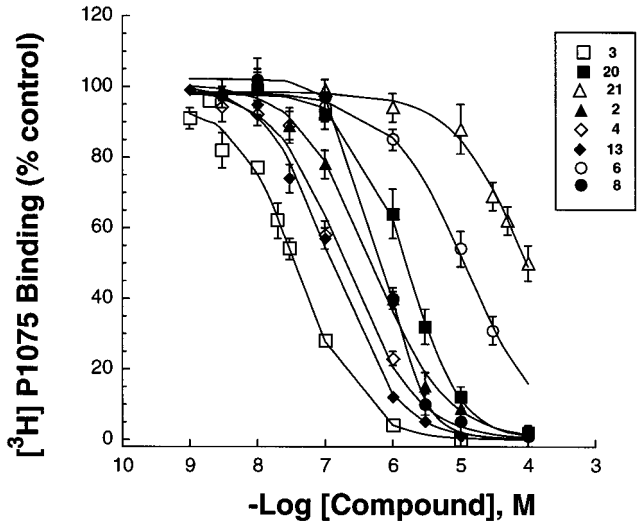


Figure 1. Competition of K_{ATP} openers for the [^{3}H]P1075 binding sites on cardiac membranes. Membranes were incubated with [^{3}H]P1075 (14 nM) and the stated concentrations of inhibitors. Binding was assessed as described and calculated as percent control. Result represent mean ± SEM of three to seven experiments.

by trypsin treatment. The binding was insensitive to calcium channel blockers, β-adrenergic antagonists, and several other classes of pharmacological agents. These characteristics are similar to those described for binding in skeletal muscle membranes.[5]

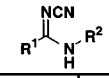
The structure−activity relationships for displacement of radioligand were determined by competition experiments using cromakalim (**1**), pinacidil (**2**) and its analogues, and the K_{ATP} blocker glyburide. The data on selected compounds is shown in Figure 1 and the K_i values for all compounds are given in Table 2. As expected, **3** is the most potent inhibitor and its calculated K_i value (27 nM) is similar to the K_d (39 nM) determined from saturation analyses and kinetic determinations. Pinacidil is 10-fold less potent than **3**. The 4-cyano analogue (**4**) of pinacidil is about 4-fold less potent than **3**. The size of the alkyl side chain is critical to potency as the methyl (**5**) and isopropyl (**6**) analogues are less potent than **4**. Replacement of the alkyl side chain with other bulky groups (**7**, **8**) maintains some inhibitory potency. The nature and the position of the electron-withdrawing group on the aromatic ring appears to be crucial as the unsubstituted (**9**), the 3-cyano (**10**), and the sulfonamido analogues (**11**, **12**) are less potent than **4**. Replacement of the cyano group in **4** with iodine maintains potency either with (**14**) or without an additional azido (**13**) substituent. The binding is stereoselectively inhibited by the benzopyran K_{ATP} opener cromakalim as its 3*S*,4*R*-enantiomer **20** (K_i = 1.1 μM) is 70-fold more potent than the 3*R*,4*S*-enantiomer **21** (K_i = 72 μM). The slope factors for all these compounds are close to unity indicating competitive displacement.

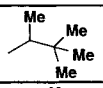
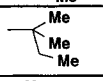
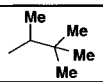
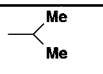
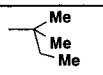
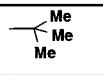
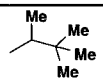
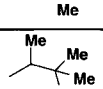
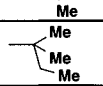
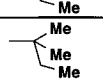
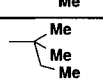
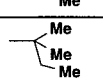
The classical K_{ATP} blocker glyburide which is known to inhibit pharmacological effects of K_{ATP} openers inhibits binding of **3** to cardiac membranes with a K_i value of 130 nM and a slope factor of 0.67 ± 0.15 nH. Analysis of the slope factor which is significantly less than unity by a two-site binding model indicates putative high- and low-affinity binding components. The high-affinity binding sites have an apparent K_i value of 70 nM which represents 70%, of the specific binding. The low-affinity sites have an apparent K_i value of 4.3 μM. The slope factor of less than unity for glyburide indicates either heterogeneous binding or a negative allosteric interaction with the binding sites for compound **3**. These data suggest that glyburide antagonizes the pharmacological effects of K_{ATP} openers in the heart by binding to a site that is in some manner associated with those occupied by K_{ATP} openers. Previous studies using intact tissues have reported similar observations for displacement of **3** by glyburide.[3]

To investigate the relevance of binding affinities to pharmacological activity in the myocardium, we compared binding affinities to cardioprotective potencies as measured by EC_{25} values for increase in time to contracture in isolated globally perfused rat hearts.[11] The EC_{25} value is defined as the concentration necessary to cause increase in time to contracture by 25% over baseline value.[11] Time to contracture is defined as the time necessary during total global ischemia to increase end diastolic pressure by 5 mmHg. In general, compounds (**2**−**4**, **7**, **13**, **14**) that are potent cardioprotectants are also potent in displacing **3** from its binding sites. The analogues (**5**, **6**, **11**, **12**) with poor cardioprotective potencies are also weak in inhibiting radioligand binding to cardiac membranes. For a correlation plot, we excluded analogues for which potency values could not be quantitated. Thus, 10 analogues with a 30-fold difference in potencies could be used for a quantitative correlation between cardioprotective potencies and binding affinities.

When the EC_{25} values for cardioprotective potencies were plotted against the K_i values for displacement of the radioligand, we obtained a good correlation coefficient (r^2 = 0.88) (Figure 2). If we take into account the analogues with poor potencies which could not be included in the correlation graph, the overall correspondence between cardioprotective potencies and binding affinities is excellent. These results suggest that binding sites in cardiac membranes may be related to those mediating cardioprotective activity of K_{ATP} openers. The cardiac membranes used in the present study were P3 pellets which contain sarcolemmal and mitochondrial membranes (data not shown). It is presently uncertain which organelle provided the binding

Table 2. K_i Values for [^{3}H]P1075 Binding Sites in Canine Cardiac Membranes, Cardioprotective and Vasorelaxation Potencies of K_{ATP} Openers



Compound	R^1	R^2	Canine cardiac [^{3}H]P1075 sites, K_i (nM)[a]	Hill Coefficient nH	TTC in Rat Hearts EC_{25} (nM)[b]	Vasorelaxation Potency IC_{50} (nM)[c]
2 (Pinacidil)			320±60	0.86±0.09	1200	59.6±8.1 (n=8)
3 (P1075)			27±5	1.17±0.15	60	4.4±0.6 (n=12)
4			99±20	0.75±0.2	390	30.3±5.2 (n=8)
5		Me	>30,000		>10,000	130000±23000 (n=4)
6			7300±1,900	0.81±0.13	>10,000	19000±1000 (n=4)
7			160±40	0.99±0.20	400	130±10 (n=4)
8			480±170	1.15±0.15	1380	530±70 (n=4)
9			680±25	1.04±0.03	1290	980±160 (n=4)
10			350±90	0.67±0.09	1460	190±10 (n=4)
11			>30,000		>10,000	12800±5700 (n=4)
12			>30,000		>10,000	>30 μM (n=3) limit of sol
13			74±8	0.76±0.06	140	1.4±0.4 (n=4)
14			57±17	0.82±0.04	39	
20 Cromakalim (3S,4R)			1100±200	1.5±0.61	2300	40.1±7.6
21 Cromakalim (3R,4S)			72000±3300	1.06±0.08	>30,000	6500±2400
Glyburide			130±40	0.67±0.15		

[a] K_i values were calculated from IC_{50}'s determined by displacement of [^{3}H]P1075 from canine cardiac membranes and Hill coefficients for competition curves were calculated by curve fitting of data to a single binding site model. [b] EC_{25} value is the concentration necessary to increase time to the onset of contracture (TTC) in globally ischemic rat hearts. [c] IC_{50} value is the concentration necessary for relaxation of the methoxamine contracted rat aorta by 50%.

sites for **3**. There is some evidence to suggest that the mechanism of cardioprotection by K_{ATP} openers does not involve sarcolemmal membrane binding sites.[12] A mitochondrial site of action is proposed for cardioprotection by K_{ATP} openers based on some preliminary studies in semipurified mitochondrial preparations.[13] Although preliminary studies to identify binding sites in purified mitochondrial membranes were unsuccessful, further work is necessary to identify the organelle that support binding in cardiac membranes.

We also attempted to correlate the binding affinities in myocardial membranes with vasorelaxant potencies. The IC_{50} values for vasorelaxant potencies were determined by relaxation of the methoxamine constricted rat aorta, as described previously.[7] The IC_{50} is defined as the concentration necessary to cause relaxation of the precontracted rat aorta by 50%. As shown in Table 2, there is 5 orders of magnitude difference in vasorelaxant potencies with compound **13** being the most potent (IC_{50} = 1.4 nM) and compound **5** being the least potent (IC_{50} = 130 μM). When the IC_{50} values for vasorelaxation were plotted against the K_i values for binding affinities, a weak correlation is obtained (r^2 = 0.71) (Figure 3). These data indicate that the binding sites for K_{ATP} openers in the cardiac membranes might be distinct from those mediating vasorelaxation in rat aorta. These results are supported by numerous studies indicating the existence of distinct structure−activity relationships for vasorelaxant and cardioprotective potencies of K_{ATP} openers.[7] It is also possible that the benzopyran derivatives and pinacidil analogues express their vasorelaxing effects by binding to distinct sites in the smooth muscle

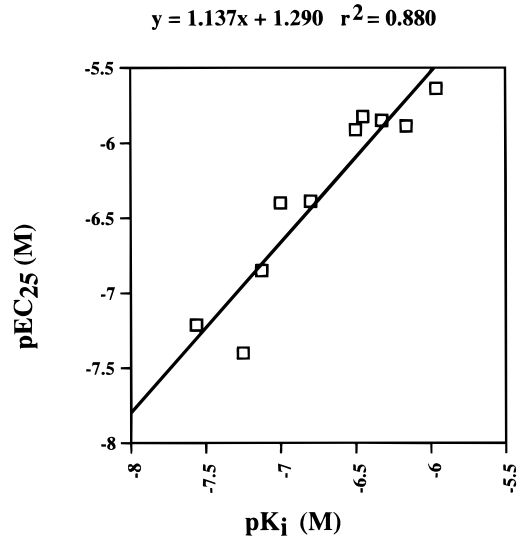


Figure 2. Correlation between binding affinities (K_i's) and cardioprotective potencies as measured by EC_{25} values for the increase in time to contracture for **2**−**4**, **7**−**10**, **13**, **14**, and **20**. A good correlation ($r^2 = 0.88$) was obtained for the specified compounds.

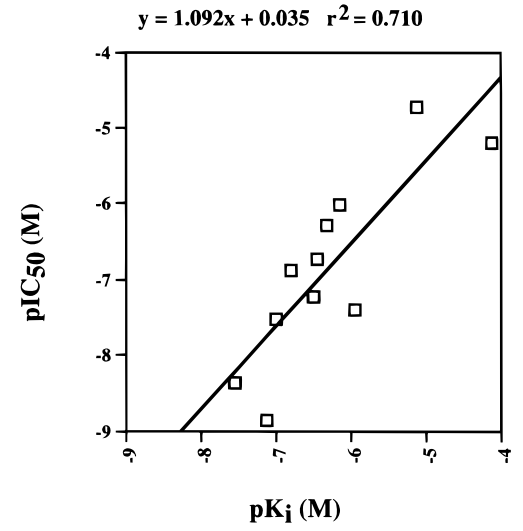


Figure 3. Correlation between binding affinities (K_i's) and vasorelaxation potencies as measured by IC_{50} values for relaxation of the precontracted rat aorta for **2**−**4**, **6**−**10**, **13**, **20**, and **21**. A weak correlation ($r^2 = 0.71$) was obtained for the specified compounds.

as a somewhat improved correlation coefficient ($r^2 = 0.85$) is obtained by excluding cromakalim enantiomers **20** and **21** from the correlation plot.

Using the pinacidil analogue **3** as a radioligand, we have demonstrated the existence of binding sites for K_{ATP} openers in canine cardiac membranes. The key to the identification of binding sites is the presence of nucleotides (e.g., MgATP) in the binding medium. Using a limited number of mostly pinacidil analogues, we have shown that there is a good correlation between binding affinities in cardiac membranes and cardioprotective potencies. A reasonably good correlation is also seen between binding affinities in canine cardiac membranes and smooth muscle relaxing potencies for the pinacidil class of compounds, although the correlation is much weaker if we include benzopyranyl K_{ATP} openers. As far as we know, this study represents the first attempt to correlate binding affinities in membrane preparations with pharmacological activities of K_{ATP} openers. Despite a good correlation, it is premature to speculate that the binding sites in cardiac membranes are relevant to functional potencies (e.g., cardioprotection) of K_{ATP} openers. However, these results are highly significant as they provide an important step forward in understanding the mechanism of action of K_{ATP} openers at the molecular level. K_{ATP} is thought to be composed of at least two distinct subunits: sulfonylurea receptor and an inward rectifier protein.[14] The binding conditions reported herein would help unravel the site of action of K_{ATP} openers using purified membrane preparations containing both the sulfonylurea receptor and the inward rectifier protein. Further, our work should facilitate the identification of binding sites in membrane preparations from other tissues and subcellular compartments where K_{ATP} openers are known to express pharmacological activity. The establishment of high throughput binding assays using membranes preparations should facilitate the identification of structurally distinct compounds with tissue selectivity.

Supporting Information Available: Experimental details, including NMR, MS, and elemental analysis (3 pages). Ordering information is given on any current masthead page.

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(8) Preparation of canine cardiac membranes: Dogs of either sex were anesthetized with Na pentobarbital (35 mg/kg) and their hearts rapidly removed and placed in ice-cold saline. The ventricles were dissected free of atria, fat, and vessels and weighed (80−100 g). The ventricles were minced and placed in ice-cold 250 mM sucrose and 50 mM Tris-HCl, pH 7.4 (1:9 w/v). The tissue was homogenized four times in a Waring blender at high speed for 20 s each. This homogenate was then further homogenized with a Brinkman polytron 3 × 10 s at setting 7. The homogenate was centrifuged at 1100*g* for 10 min and the pellet was designated P1. The supernatant was filtered through two layers of cheesecloth and centrifuged at 12800*g* for 10 min (P2). The supernatant was centrifuged at 105000*g* for 35 min and the pellet (P3) resuspended in 50 mL of 0.6 M KCl, 4 mM imidazole, pH 7.4, incubated on ice for 1−3 h and then centrifuged at 100000*g* for 35 min. The pellet was resuspended in sucrose/Tris buffer at a protein concentration of approximately 10 mg/mL and stored frozen at −80 °C until use.

(9) [3H]P1075 binding assay: Assays were conducted in 12 × 75 mm tubes in a total volume of 1 mL. The assay buffer consisted of 240 mM sucrose, 3 mM sodium orthovanadate, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, and 2 mM MgATP. The nucleotides (sodium or trilithium salts), **3** (14 nM, 51−61 Ci/mmol), and inhibitors were incubated with membranes (500−700 μg) for 60 min. Nonspecific binding was defined with 10 μM nontritiated **3**. Bound and free radioligand were separated by filtration through a GF/C glass fiber filter on a Brandel cell harvester with 3 × 2 mL washes of 50 mM Tris, pH 7.4, at 4 °C. The filters were transferred to 6 mL plastic scintillation vials, soaked overnight in 5 mL of scintillation fluid (Packard's Opti-fluor), and counted in a Packard liquid scintillation counter.

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