Pharmacological Characterization of Novel Cyanoguanidines as Vascular $K_{\text{ATP}}$ Channel Blockers

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ABSTRACT
$K_{\text{ATP}}$ blockers derived from cyanoguanidine $K_{\text{ATP}}$ opener (P1075) chemistry were characterized in isolated rabbit mesenteric artery and evaluated functionally by their ability to antagonize maximal relaxation induced by pinacidil (1 mM) of norepinephrine (5 μM) contraction. PNU-89892, PNU-97025E and PNU-99963 were identified as $K_{\text{ATP}}$ blockers with IC$_{50}$ values of 880, 83 and 18 nM, respectively. Studies with selected chiral compounds demonstrated that the (R)-enantiomers were more potent as $K_{\text{ATP}}$ blockers than the (S)-enantiomers. Further studies demonstrated that PNU-99963 (1) inhibited relaxations by other $K_{\text{ATP}}$ openers, such as cromakalim (0.5 μM) and minoxidil sulfate (5 μM); (2) was more potent than the other known vascular $K_{\text{ATP}}$ blockers (glyburide and PNU-37883A); and (3) acted as a $K_{\text{ATP}}$ blocker in isolated rat aorta as well as dog coronary artery. PNU-99963 actions were selective because PNU-99963 (100 nM) was without any inhibitory effect on relaxations induced by forskolin (0.5 μM), nitroglycerin (1 μM), D600 (25 or 500 nM) or 15 mM K$^+$-induced relaxations of NE contractions in K$^+$-free PSS. The discovery of $K_{\text{ATP}}$ blockers and openers from the same chemical series is a first for the $K_{\text{ATP}}$ channel field. The close structural similarity between P1075 ($K_{\text{ATP}}$ opener) and PNU-99963 ($K_{\text{ATP}}$ blocker), stereospecificity of action and potency and selectivity all suggest that these molecules may prove to be valuable tools in understanding the structure and function of the $K_{\text{ATP}}$ channel complex in vascular smooth muscle.

It is well established that there exists a structurally diverse group of compounds that produce vasorelaxation via activation of vascular $K_{\text{ATP}}$ channels (Edwards and Weston, 1993; Triggle, 1990). The most well known of these $K_{\text{ATP}}$ opener vasodilators include clinically used antihypertensives such as minoxidil ([via its active metabolite, minoxidil sulfate] and pinacidil, as well as experimental drugs such as cromakalim (Cook and Quast, 1990; Edwards and Weston, 1993; Primeau and Butera, 1995). One critical experimental tool used in the characterization of these compounds as vascular $K_{\text{ATP}}$ openers has been the glyburide, a potent $K_{\text{ATP}}$ blocker. Glyburide, a sulfonylurea, is the most potent blocker of $K_{\text{ATP}}$ channels in a variety of cell systems including vascular smooth muscle (Aschcroft, 1988; Edwards and Weston, 1993). In addition to glyburide, several other structurally distinct compounds have also been claimed to be $K_{\text{ATP}}$ blockers (Edwards and Weston, 1993). For example, we have reported the actions of PNU-37883A, a guanidine, as a structurally novel $K_{\text{ATP}}$ blocker in vascular smooth muscle (Meisher et al., 1993a). PNU-37883A is unique because it is a rather selective $K_{\text{ATP}}$ blocker for the vasculature (Guillemare et al., 1994; Meisher et al., 1993a). Although PNU-37883A is more vascular selective than glyburide as a $K_{\text{ATP}}$ blocker, it is ~10-fold less potent than glyburide (Meisher et al., 1993b; Ohrnberger et al., 1993).

We have been interested in identifying novel and more potent vascular $K_{\text{ATP}}$ blockers that may become useful tools in furthering the understanding of mechanisms of $K_{\text{ATP}}$ modulation in vascular smooth muscle. This is because vascular smooth muscle is the primary in vivo tissue target for the actions of $K_{\text{ATP}}$ openers. In the present study, we describe a new chemical class of compounds, cyanoguanidines, as vascular $K_{\text{ATP}}$ blockers with two distinctly novel features: i) the cyanoguanidine $K_{\text{ATP}}$ blockers described in this study were derived from the cyanoguanidine based $K_{\text{ATP}}$ openers, such as pinacidil and P1075 and ii) we have identified a cyanoguanidine (PNU-99963) with a pharmacological potency greater than that of glyburide for vascular $K_{\text{ATP}}$ blockade.

Materials and Methods

General. The majority of experiments were carried out using isolated rabbit mesenteric artery. Adult New Zealand White rabbits (2–3 kg) were anesthetized with Metofane (methoxyflurane) and killed by exsanguination. The superior mesenteric artery was excised rapidly and placed in warm (37°C), oxygenated PSS and cleaned of fat and connective tissue. The vessels were cut into 2–3-mm-wide rings for use in isolated tissue bath experiments as described previously (Higdon et al., 1997; Ohrnberger et al., 1993).

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ABBREVIATIONS: $K_{\text{ATP}}$, ATP-sensitive K$^+$ channel; CRC, concentration-response curve; NE, norepinephrine; PSS, physiological salt solution; RMA, rabbit mesenteric artery.
The tissues were suspended under 1 g resting tension and allowed to equilibrate 60 min in warm PSS before the experiments were started.

Inhibitory effects of K\textsubscript{ATP} blockers on pinacidil-induced relaxation. Ability of cyanoguanidines to inhibit the relaxant response to a maximally effective concentration of pinacidil was used as a functional indicator of their K\textsubscript{ATP} blocking effect. This protocol has been developed in this laboratory and has been extensively used to characterize K\textsubscript{ATP} blocking effects of glyburide and PNU-37883A (Meišeri et al., 1991; Ohrmberger et al., 1993). Briefly, a control pinacidil (1 μM) relaxation was first studied on NE (5 μM) precontracted tissue. Tissues showing <80% relaxation to pinacidil within 10 to 15 min were not used. Tissues were washed with PSS, returned to resting tension and pretreated with K\textsubscript{ATP} blockers for 45 to 60 min. Tissues were then recontracted with NE, and pinacidil-induced relaxation was again studied. The degree of K\textsubscript{ATP} channel-blocking activity was determined by comparing pinacidil relaxation before and after the test compound in the same tissue. A CRC for a KATP blocker was generated using several concentrations of a compound. A given tissue was exposed to only a single concentration of a blocker. K\textsubscript{ATP} blockers studied were structurally very similar to P1075 (a K\textsubscript{ATP} opener), relaxation responses to P1075 on NE-precontracted RMA were generated for comparison. Cumulative relaxation CRCs were generated for P1075 (5–100 nM).

Studies with PNU-99963. A series of P1075 analogs were synthesized for initial screening (Humphrey et al., 1994). From among the K\textsubscript{ATP} blockers studied, PNU-99963 was found to be the most potent compound, so subsequent detailed experiments were carried out to further characterize PNU-99963. In one series of experiments, cumulative relaxation CRCs were generated for pinacidil (0.05–10 μM) with and without PNU-99963 pretreatment at the maximally effective concentration of 100 nM. In another series of experiments, the ability of PNU-99963 (100 nM) to inhibit relaxations by other K\textsubscript{ATP} blockers was studied using cromakalim (0.5 μM) and minoxidil sulfate (5 μM). The concentrations of K\textsubscript{ATP} openers chosen were based on our previous studies in this preparation and have been shown to produce maximal relaxation (>80%) within an optimal time course of 15 min (Meišeri et al., 1993b).

Selectivity of PNU-99963. Three types of experiments were carried out to investigate the pharmacological selectivity of PNU-99963 in isolated RMA. First, PNU-99963 (100 nM) was tested against relaxations produced by forskolin (0.5 μM), a cyclic AMP activator, and by nitroglycerin (1 μM), a cyclic GMP activator. Forskolin and nitroglycerin each produced a similar degree of maximal relaxation, with an inhibitory IC\textsubscript{50} (nM) of 80 mM PSS was prepared by equimolar replacement of NaCl with KCl. Compound sources were as follows: norepinephrine HCl and ouabain were from Sigma Chemical (St. Louis, MO). Nitroglycerin (Tridil) was from Dupont (Wilmington, DE). Forskolin was from Calbiochem (San Diego, CA). D600 HCl was from AG Knoll Pharmaceuticals (Orange, NJ). PNU-46619, P1075 (PNU-83757), pinacidil HCl, cromakalim, minoxidil sulfate, PNU-89692, PNU-94563, PNU-94126, PNU-94158, PNU-94750, PNU-96179, PNU-96293, PNU-99963 and PNU-97025E were from Pharmacia & Upjohn. All drugs were prepared as 10 mM stock solution using water or, if necessary, DMSO.

Data collection and statistics. Computerized data acquisition system and customized spreadsheets used for the analysis of recordings of contractions and relaxations have been described before (Khan et al., 1993). All data are expressed as mean ± S.E.M. (n). EC\textsubscript{50} values were calculated using NLIN2 (SAS based program). EC\textsubscript{50}/IC\textsubscript{50} was defined as the concentration of drug that produces 50% of the maximum response. CRCs were generated using SLIDE-WRITE programs. Student's t test was used at P < .05 for statistical significance.

Results

Identification of cyanoguanidine K\textsubscript{ATP} blockers. Initially, a range of structurally diverse analogs of P1075 were synthesized and screened for potential K\textsubscript{ATP} opening activity using precontracted RMA. Those proving to be fairly inactive as K\textsubscript{ATP} openers were selected for testing as potential K\textsubscript{ATP} blockers on the basis of an idea that chemical modification may have converted openers into blockers. This approach yielded PNU-89692 as the first P1075-based cyanoguanidine acting as a K\textsubscript{ATP} blocker. The CRC for PNU-89692 as a K\textsubscript{ATP} blocker is shown in figure 1. Also shown in this figure is the CRC for P1075 as a K\textsubscript{ATP} opener. PNU-89692 (0.1–5 μM) showed K\textsubscript{ATP} blocking activity against pinacidil maximal relaxation, with an inhibitory IC\textsubscript{50} (μM) of 0.86 ± 0.04. PNU-89692 at 5 μM caused close to 90% inhibition of pinacidil maximal relaxation. P1075 is a potent K\textsubscript{ATP} opener vasodilator showing relaxation in the range of 5 to 100 nM with a relaxation EC\textsubscript{50} (nM) = 21 ± 1.6.

Having identified PNU-89692 as the first cyanoguanidine K\textsubscript{ATP} blocker, our next series of experiments were targeted at identifying more potent cyanoguanidine blockers that were structurally based on PNU-89692. Figure 2 shows a comparison of three cyanoguanidines as K\textsubscript{ATP} blockers. In comparison to PNU-89692, PNU-97025E was ~10-fold more potent with an inhibitory IC\textsubscript{50} (μM) of 0.083 ± 0.005. A further structural modification yielded PNU-99963, which showed very high activity as a K\textsubscript{ATP} blocker. PNU-99963 was active in the concentration range of 5 to 100 nM with an inhibitory IC\textsubscript{50} (nM) = 18 ± 2.

Cyanoguanidine K\textsubscript{ATP} blockers stereoselectivity. It is known that cyanoguanidine K\textsubscript{ATP} opener vasodilators...
demonstrate stereoselectivity, with the (R)-enantiomer being significantly more potent than the (S)-enantiomer (Cook and Quast, 1990). Thus, we investigated whether the cyanoguanidine KATP blockers also exhibit stereoselectivity. Data with selected compounds are presented in figure 3. Two chiral compounds, PNU-94563 (top) and PNU-94750 (bottom), showed maximal KATP blocking activity at 5 \( \mu \)M. In each case, the (R)-enantiomers (PNU-94126: top, and PNU-96293: bottom) were significantly more potent than the corresponding racemates, whereas the (S)-enantiomers (PNU-94158: top, and PNU-96179: bottom) were found to be inactive as KATP blockers in comparison with their corresponding racemates.

Further studies with PNU-99963. Because PNU-99963 was found to be a highly potent blocker, studies were carried out to further characterize this compound in RMA. Figure 4 (top) shows the results of an experiment in which the effect of PNU-99963 (100 nM) pretreatment on pinacidil cumulative relaxation CRC was investigated. In NE-precontracted RMA, pinacidil produced relaxation CRC with an EC50 of 2.7 \( \pm \) 0.09 \( \mu \)M. Maximum relaxation of >80% by 1 \( \mu \)M pinacidil in control tissues was reduced to <20% in PNU-99963 pretreated tissues.
Figure 4 (bottom) shows that PNU-99963 (100 nM) was effective in inhibiting relaxations not only by pinacidil but also by the other chemical classes of KATP openers such as cromakalim and minoxidil sulfate. In each case, maximal relaxation by a KATP opener was significantly attenuated, and the degree of blockade induced by PNU-99963 in all three cases was $80\%$.

Selectivity of PNU-99963. Three types of studies were done to establish the pharmacological selectivity of PNU-99963 actions. Figure 5 (top) shows that PNU-99963 (100 nM) did not produce any significant attenuation of relaxation by forskolin (0.5 mM) or nitroglycerin (1 mM). The concentrations of forskolin and nitroglycerin used were equieffective to pinacidil in producing maximal relaxations. Figure 5 (bottom) shows the results of an experiment designed to study direct functional effects of PNU-99963 on voltage-gated calcium channels. In RMA precontracted with 80 mM KCl, PNU-99963 was without any significant ($P < .05$) effect on relaxations produced by D600 at low concentration (25 nM) or high concentration (500 nM). Finally, the third experiment was designed to investigate potential inhibitory effects of PNU-99963 on Na$^+$-K$^+$ ATPase pump activity. In RMA precontracted with NE in K$^+$-free PSS, addition of 15 mM KCl produced a relaxation that was effectively eliminated by ouabain pretreatment (fig. 6: tracings at the top and bar graph at the bottom). In fact, ouabain pretreatment converted 15 mM K$^+$-induced relaxation into a contraction. In contrast, the K$^+$-induced relaxation remained completely unaffected by pretreatment with 100 nM PNU-99963.

PNU-99963 induced KATP blockade in dog coronary artery and rat aorta. Experiments were designed to test if PNU-99963 was also an effective KATP blocker in different vasculature from different species. RMA was compared with dog coronary artery and rat aorta, tissues in which KATP openers and blockers have been extensively studied. As shown in figure 7, PNU-99963 was an effective KATP blocker in all three tissues. The threshold for KATP blocking activity was $10$ nM in all three tissues, and all three tissues exhibited maximum blockade by PNU-99963 at 100 nM. Dog coronary artery appeared slightly more sensitive, demonstrat-
ing a higher degree of blockade at 20 nM PNU-99963. 
Approximate IC50 values for PNU-99963 for KATP blockade in all three tissues ranged from 15 to 20 nM. Similar results were found with another blocker PNU-97025E (tested at 10, 30, 100 and 300 nM), which also showed similar KATP blockade in all three vascular preparations (data not shown). 

**Comparison of PNU-99963 in RMA with glyburide and PNU-37883A.** Figure 8A shows comparative CRCs for functional KATP blockade by PNU-99963 (cyanoguanidine), glyburide (sulfonylurea) and PNU-37883A (guanidine). All three CRCs were generated using an identical protocol as described in the previous sections. As can be seen, PNU-99963 is clearly the most potent vascular KATP blocker identified to date. Figure 8B shows the similarity in structures of PNU-99963 (cyanoguanidine KATP blocker) and P1075 (a cyanoguanidine KATP opener). The CRC for P1075 as a KATP opener (left y axis) and that for PNU-99963 as a KATP blocker (right y axis) are superimposable. The IC50 values for PNU-99963 as a blocker and the EC50 for P1075 as an opener are also very similar (18 vs. 21 nM, respectively).

**Discussion**

This report provides the first pharmacological characterization of a structurally novel and potent vascular KATP blocker. Two novel features of this report are (1) the cyanoguanidine KATP blockers described in this study were derived from the potent cyanoguanidine KATP opener P1075.
and (2) PNU-99963 has a pharmacological potency greater than that of glyburide for vascular K$_{ATP}$ blockade. These observations are discussed below.

Cyanoguanidine K$_{ATP}$ openers, as represented by pinacidil and P1075, are well known vasodilator antihypertensives. Extensive published work shows that these compounds preferentially and selectively activate vascular K$_{ATP}$ channels, produce hyperpolarization, cause vasorelaxation and lower blood pressure (Buckingham, 1990; Cook and Quast, 1990). Two events led us to investigate cyanoguanidine molecules as potential K$_{ATP}$ blockers. First, as part of a drug discovery research program at Pharmacia & Upjohn, many novel analogs were synthesized from the cyanoguanidine (pinacidil/P1075) and benzopyran (cromakalim) series of K$_{ATP}$ openers and analyzed for vascular K$_{ATP}$ opening activity. Second, it was observed that while K$_{ATP}$ openers possessed vasorelaxant and in vivo hypotensive properties, the known K$_{ATP}$ blockers (glyburide and PNU-37883A) have significant euclidean diuretic activity in vivo (Clark et al., 1993; Humphrey et al., 1995; Perricone et al., 1994). Thus, it was considered a possibility that weakly active or inactive K$_{ATP}$ openers may have been converted into K$_{ATP}$ blockers due to structural modification and might be worth testing for K$_{ATP}$ blocking activity. A systematic screening of inactive cyanoguanidine K$_{ATP}$ openers identified PNU-89692 first as a weak in vivo diuretic and subsequently as a weak in vitro vascular K$_{ATP}$ blocker. A follow-up collaborative effort involving directed synthesis based on PNU-89692 and study of vascular K$_{ATP}$ blocking activity resulted in the identification of potent cyanoguanidine K$_{ATP}$ blockers, represented by PNU-97025E and PNU-99963.

The in vitro vascular preparation of isolated rabbit mesenteric artery used here has been extensively used for the study of known K$_{ATP}$ openers as well as blockers (Gojkovic and Kazic, 1994; Ohrnberger et al., 1993; Post and Jones, 1991; Silberberg and Van Breemen, 1992; Standen et al., 1989; Quayle et al., 1995). We have taken advantage of this well established experimental model to characterize new K$_{ATP}$ blockers. As has been discussed previously, functional studies require careful titration of optimal concentrations of drugs used (Meisheri et al., 1993b). Pronounced attenuation of pinacidil relaxation by PNU-99963 suggested that this compound is working via inhibiting K$_{ATP}$ channels. Due to the complexity of the pharmacological studies in intact preparations to define cellular mechanisms, it was considered critical to carry out experiments to exclude possible nonspecific effects. First, antagonism by PNU-99963 of pinacidil relaxation could be due to nonspecific depolarization caused by, for example, inhibition of Na$^+$-$K^+$ ATPase. Our data with K$^+$-induced relaxation, which is mediated via the K$^+$-induced activation of Na$^+$-$K^+$ ATPase (Webb and Bohr, 1978), shows that this is not the case. Second, because pinacidil induces relaxation by causing hyperpolarization-mediated inhibition of voltage-gated Ca$^{2+}$ channels, PNU-99963 could directly modulate Ca$^{2+}$ channels in a manner that would antagonize pinacidil relaxation. This also is not the case because PNU-99963 did not modify relaxation of high-K$^+$ contractions by D600, a Ca$^{2+}$ antagonist. Finally, PNU-99963 could be interfering with other Ca$^{2+}$ homeostasis mechanisms or Ca$^{2+}$ sensitivity mechanisms. It is well known that both cAMP and cGMP pathways in smooth muscle utilize multiple Ca$^{2+}$ homeostasis and sensitivity mechanisms. The preferential inhibition by PNU-99963 of pinacidil relaxation in comparison to relaxations by forskolin (a cAMP activator) or nitroglycerin (a cGMP activator) strongly suggest that PNU-99963 is targeting a specific mechanism. Furthermore, nitroglycerin-type cGMP activators have recently been shown to work via activating vascular maxi-K$^+$ or BK channels. Thus, the data presented collectively lead to our interpretation that the primary target of PNU-99963 in vascular smooth muscle is the K$_{ATP}$ channel mechanism. However, further investigation and confirmation of the mechanism of action of PNU-99963 would require direct electrophysiological studies as well as isotopic ion flux studies in vascular smooth muscle.

Several interesting features of the cyanoguanidines as K$_{ATP}$ blockers were discovered. First was the demonstration of stereoselectivity. Two of the racemates tested in this study showed that, in each case, the (R)-enantiomer showed greater potency than the (S)-enantiomer as a blocker. Interestingly, this is also the case with cyanoguanidine K$_{ATP}$ openers because the (R)-enantiomer of pinacidil is 6- to 8-fold more potent as a vasodilator than the (S)-enantiomer (Cook and Quast, 1990). Second, it was found that the cyanoguanidine blockers are not only effective in inhibiting K$_{ATP}$, mediated vasorelaxation by cyanoguanidine openers, but they also inhibit relaxations by other, structurally diverse K$_{ATP}$ openers such as cromakalim and minoxidil sulfate. This suggests that PNU-99963 is targeting a step that is common to the pathway(s) used by all different K$_{ATP}$ blockers. A similar observation regarding other K$_{ATP}$ blockers such as glyburide and PNU-37883A has also been made (Ohrnberger et al., 1993). Third, our data show that PNU-99963 acts as a K$_{ATP}$ blocker not only in rabbit mesenteric artery but also in isolated rat aorta as well as dog coronary artery. Rat aorta and dog coronary artery are two other preparations in which a large amount of work with K$_{ATP}$ blockers and blockers has been carried out (Cook and Quast, 1990). Thus, it was important to demonstrate that different vascular preparations (mesenteric, aorta, coronary) from different species (rat, rabbit, and dog) are similar in their responses to PNU-99963 as a K$_{ATP}$ blocker. Finally, comparative data show that under identical experimental conditions, PNU-99963 is the most potent vascular K$_{ATP}$ blocker in comparison with PNU-37883A and glyburide.

There are two areas that may prove fruitful for further investigations. First is the question of vascular vs. nonvascular tissue selectivity. It would be of interest to know the relative selectivity of cyanoguanidine K$_{ATP}$ blockers on vasculature vs. pancreatic $\beta$ cell vs. cardiac tissue vs. brain. No systematic data in cell types other than the vasculature are currently available with PNU-99963. The second area of interest is the biochemical mechanism by which PNU-99963 may modulate K$_{ATP}$ channels. It is of interest to note that a high-affinity receptor site for a cyanoguanidine K$_{ATP}$ opener (P1075) has been proposed and characterized in vascular smooth muscle (Quast et al., 1993). However, the functional relevance of this P1075 receptor site has been recently questioned (Higdon et al., 1997). Availability of PNU-99963 provides an opportunity for using a highly potent cyanoguanidine K$_{ATP}$ blocker as a ligand for radioisotopic studies to
identify and characterize sites involved in vascular K\textsubscript{ATP} modulation. A low-affinity sulfonylurea receptor site modulating K\textsubscript{ATP} channel activity in smooth muscle has been recently suggested (Isomoto et al., 1996; Lloffer and Quast, 1997). Similarly, a low-affinity receptor site for PNU-37883A has also been described (Guillemare et al., 1994; Meisheri et al., 1995). The relationship of PNU-99963 and related cyanoguanidine K\textsubscript{ATP} blockers to the receptor sites for sulfonylureas or guanidine K\textsubscript{ATP} blockers remains to be established.

In summary, the discovery of openers and blockers of the K\textsubscript{ATP} channel from the same chemical series represents a unique development in the K\textsuperscript{+} channel field and is equivalent to the well-known development of openers and blockers of the voltage-gated Ca\textsuperscript{2+} channels from the dihydropyridine chemistry. The close structural similarity between P1075 (K\textsubscript{ATP} opener) and PNU-99963 (a K\textsubscript{ATP} blocker), stereospecificity of action, as well as the potency and selectivity, all suggest that these molecules may prove to be valuable tools in furthering our understanding of the structure and function of the K\textsubscript{ATP} channel complex in vascular smooth muscle.

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