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Pharmacology of the K-ATP Channel Blocking Morpholinoguanidine PNU-37883A

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INTRODUCTION

The last two decades have seen enormous growth in our understanding of ion channel structure and function. In particular, adenosine-5'-triphosphate-sensitive potassium (K-ATP) channels have been intensely investigated because of their wide distribution, key role in linking cellular metabolism to membrane potential, and therapeutic potential of K-ATP modulators (4,14,36,95). Many other reports have further described how K-ATP channels help to control insulin secretion (3,24,96), vascular smooth muscle tone (31,94,109), myocardial contractility and preconditioning (5,32,37,64), and renal tubular function (46, 93,104,118). Common to these many in depth investigations has been an armamentarium of specific channel modulators, such as the K-ATP openers pinacidil, minoxidil sulfate, and cromakalim, and the K-ATP channel blockers glyburide (glibenclamide) and sodium 5-hydroxydecanoate (Fig. 1). Newly added to this arsenal is the morpholinoguanidine PNU-37883A (originally designated U-37883A), which has been shown to functionally antagonize vascular K-ATP channel openers *in vitro* and *in vivo* (78) and to block K-ATP channels in *Xenopus* oocyte follicles (41), rat renal tubules (116), vascular smooth muscle (47,122), and central neurons (67). As summarized in this review, PNU-37883A is a potent, euglycemic vascular K-ATP blocker that also exerts a K^+-sparing diuresis at higher doses. PNU-37883A thus represents a very important new experimental agent for examining K-ATP control of vascular smooth muscle tone, urinary electrolyte excretion, renin secretion, and neuronal function.

CHEMISTRY AND INITIAL DETECTION OF BIOLOGICAL ACTIVITY

The synthesis and initial detection of PNU-37883A's biological effects have been described by Perricone et al. (91). Chemically PNU-37883A is 4-morpholinecarboxamidine-*N*-1-adamantyl-*N′*-cyclohexyl hydrochloride (Fig. 1), characterized by its electro-

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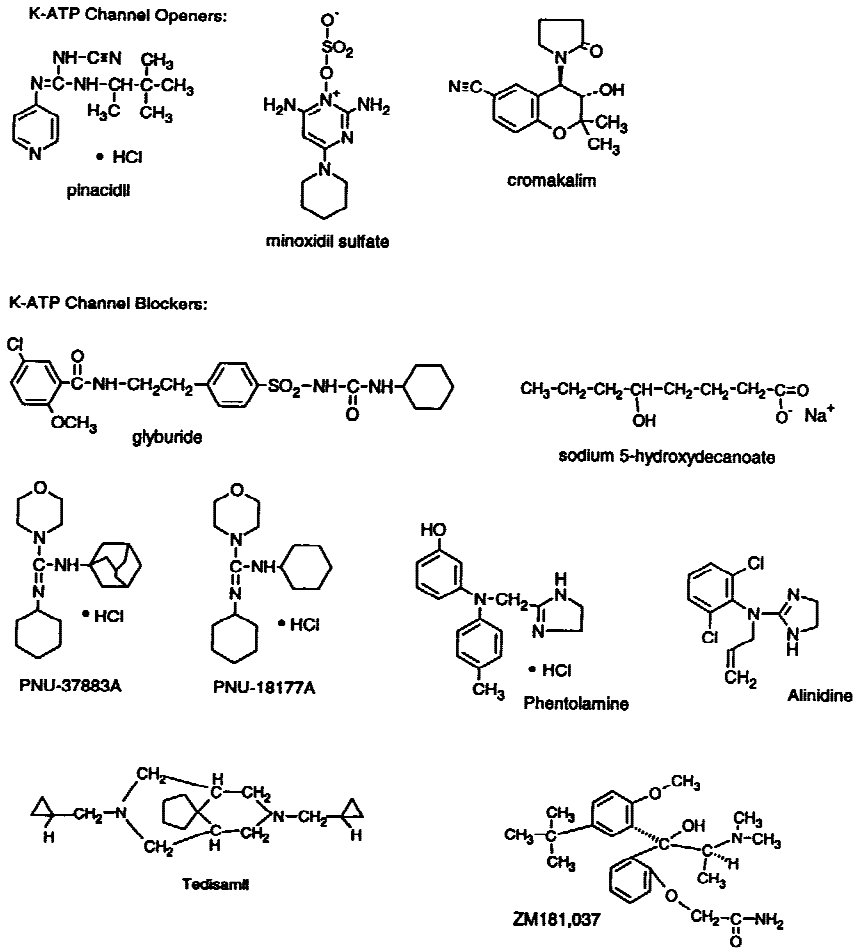

FIG. 1. Chemical structures of established K-ATP channel openers and blockers as compared with the morpholinoguanidine PNU-37883A, its dicyclohexyl precursor PNU-18177A, and several chemically unrelated agents exhibiting vascular K-ATP channel blocking effects.

negative morpholinyl oxygen and protonated central guanidine group. Despite the bulk of its lipophilic cyclohexyl and adamantyl moieties, small pockets of surface positivity are expressed at the base of each ring, enhancing the drug's aqueous solubility ($\leq$10 mg/mL). PNU-37883A was first synthesized in 1971 as an analog of PNU-18177A (Aldrich 16,320-1; Fig. 1), a diuretic lead that doubled urinary Na^+ excretion in rats without excess K^+ loss but which proved acutely toxic at higher doses (91). Relative to its precursor, PNU-37883A's slightly larger adamantyl ring enhanced its natriuretic efficacy, but the drug was never developed because of its equally profound myocardial depression near its therapeutic dose range.

Aside from the relatively eukalemic diuretic profiles of PNU-18177A and PNU-37883A, their most intriguing aspect was the ability to rapidly reverse the normally sustained hypotensive effect of the vasodilator drug candidate minoxidil. As development

proceeded with both chemical series, questions arose as to whether PNU-18177A would attenuate minoxidil's avid salt retention (126), or whether minoxidil's coronary vasodilation (56) would mitigate the morpholinoguanidines' cardiotoxicity. While formal interaction studies addressing these questions were never implemented, as shown in Fig. 2, acute i.v. PNU-18177A unexpectedly reversed minoxidil's arterial hypotension in conscious dogs, suggesting direct antagonism of the latter's peripheral vasodilation (91). A similar vascular interaction was subsequently confirmed with PNU-37883A, which reversed the effects of minoxidil in anesthetized cats (Fig. 2). The implication of these *in vivo* interactions was not fully appreciated, however, until it was later established that minoxidil's active sulfate metabolite relaxes vascular smooth muscle by opening K-ATP channels (77), thus leading to speculation that its antagonism by PNU-18177A and PNU-37883A was due to vascular K-ATP blockade.

FUNCTIONAL ANTAGONISM OF VASCULAR K-ATP OPENERS

In Vitro Blockade of K-ATP Vasorelaxation and $^{42}K^+$ Efflux

Meisheri et al. (78) first characterized PNU-37883A's functional antagonism to K-ATP channel opener-mediated vasorelaxation in the norepinephrine-contracted rabbit mesenteric artery. In this *in vitro* model, glyburide consistently blocks agents such as pinacidil, minoxidil sulfate, cromakalim, and lemakalim with IC_{50}s of 72 to 148 nM (79). In their investigations, 0.5 to 5 μM PNU-37883A likewise reversed and prevented 1 μM pinacidil's vasorelaxation with an IC_{50} of 0.78 μM (Fig. 3), thereby proving to be about 1/10

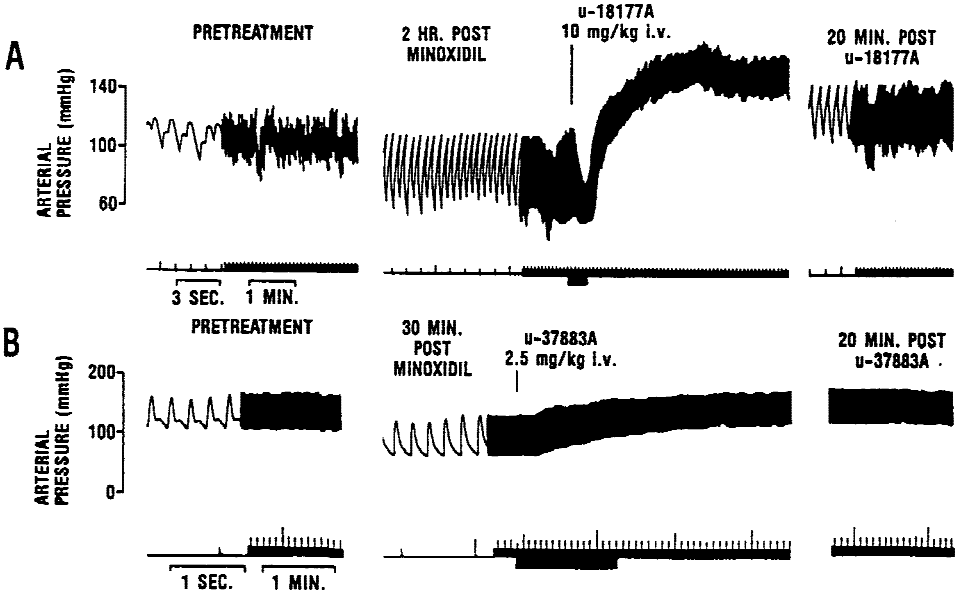

FIG. 2. Original polygraph traces showing the reversal of minoxidil vasodilation in a conscious dog (*A*) and an anesthetized cat (*B*) by PNU-18177A (10 mg/kg i.v.) and PNU-37883A (2.5 mg/kg i.v.), respectively. In both cases, at 1 mg/kg, minoxidil's normally sustained (≥24 h) hypotension was rapidly reversed, suggesting direct antagonism to its arterial vasodilation. Subsequent investigations confirmed that minoxidil's active sulfate metabolite relaxes vascular smooth muscle by opening K-ATP channels, leading to investigations demonstrating that PNU-37883A potently and selectively blocks vascular K-ATP channels.

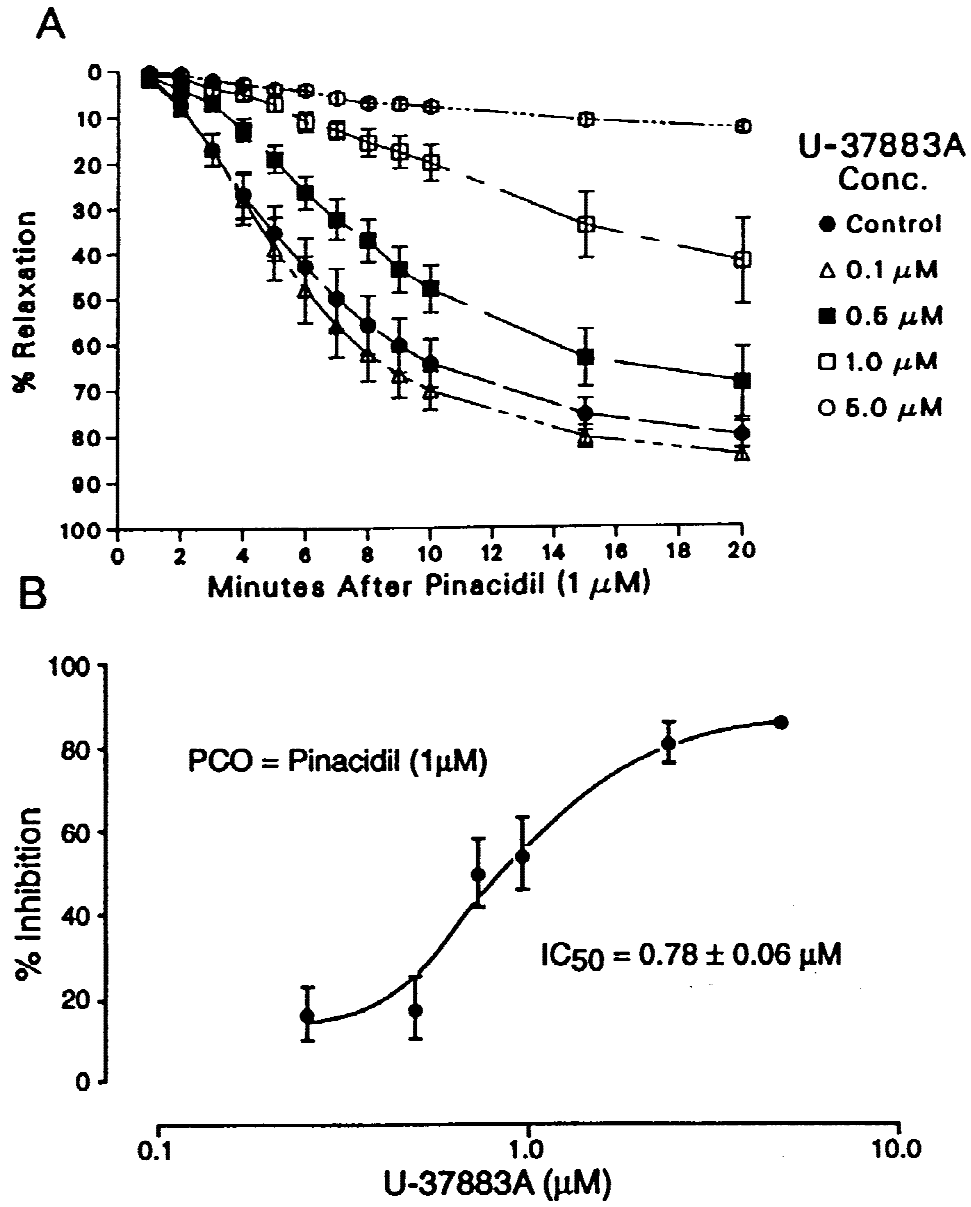

FIG. 3. PNU-37883A inhibition of 1 μM pinacidil vasorelaxation in 5 μM norepinephrine-contracted rabbit mesenteric artery (RMA). *A,* time course of 0.1 to 5 μM PNU-37883A antagonism to pinacidil (mean ± S.E. for 4 to 6 RMA rings/data point); *B,* concentration-response for PNU-37883A inhibition of pinacidil in this assay. Fifteen-min inhibitory data were converted to the percentage of inhibition based on pinacidil's control relaxation of 80 ± 3%. The computer-derived 50% inhibitory concentration (IC_{50}) for PNU-37883A was 0.78 ± 0.06 μM. From ref. 78, with permission.

as potent as glyburide. These PNU-37883A concentrations did not affect basal vascular tone and also blocked the vasorelaxant effects of 5 μM minoxidil sulfate, 0.5 μM cromakalim, and 1 μM lemakalim with IC_{50}s of 0.84, 1.4, and 1.0 μM, respectively. Only 5-min PNU-37883A preexposure was necessary for these functional K-ATP channel blocking effects as compared with 15 min for glyburide. Indicative of the drug's K-ATP selectivity, 10 μM PNU-37883A increased pinacidil's vasorelaxant EC_{50} in the rabbit mesenteric artery 32-fold, while not significantly affecting the vasorelaxant effects of a Ca^{2+} channel blocker (D-600), forskolin, or nitroglycerin. Also consistent with a K-ATP blocking mechanism, PNU-37883A prevented and blocked K-ATP channel opener-induced $^{42}K^+$ efflux (Fig. 4) but did not antagonize pinacidil or ouabain in 80 mM K^+-contracted rabbit mesenteric artery. These initial investigations thus clearly identified PNU-37883A as a selective functional antagonist of K-ATP channel opener-mediated vasorelaxation and $^{42}K^+$ efflux, which could be useful in evaluating this mechanism of modulating vascular smooth muscle tone.

In Vitro Binding and Vascular Interactions

The basis for PNU-37883A's K-ATP channel blocking effect was further probed by Oleynek and Meisheri (88), who found that 5 to 500 nM [^{3}H]PNU-37883A specifically bound to rabbit mesentery artery with a saturable K_d of 65 nM. Tritiated PNU-37883A was displaced by unlabeled drug and PNU-18177A but not by the inactive unsubstituted morpholinoguanidine analog PNU-42069D, thus demonstrating that PNU-37883A's binding to this vascular tissue was reversible and pharmacologically relevant. Remarkable synergy was also seen between PNU-37883A and glyburide, as Ohrnberger et al. (87) reported that a threshold concentration of 0.5 μM PNU-37883A potentiated glyburide's IC_{50} against pinacidil vasorelaxation 18-fold, while low-concentration glyburide (50 nM) likewise potentiated PNU-37883A's IC_{50} versus pinacidil 7-fold. Similar reciprocal potentiation was also seen against other K-ATP channel openers, independent of D600 vasorelaxation. Based on these findings, a model was proposed whereby PNU-37883A and glyburide appear to act at different sites on a common protein linked to vascular K-ATP channels, such that minimal concentrations of each significantly enhance the blocking effect of the other (87).

PNU-37883A's functional K-ATP blocking and vascular binding effects have more recently been profiled by Löffler-Walz and Quast (71) in rat aorta. In this preparation PNU-37883A and the highly efficacious loop diuretic furosemide did not alter the binding of the extremely potent pinacidil analog [^{3}H]P-1075, and high levels of PNU-37883A ($\geq$10 μM) were necessary to nonspecifically displace 3[H]glyburide. In $^{86}Rb^+$ efflux experiments, PNU-37883A was inactive against basal activity but markedly inhibited P-1075-stimulated $^{86}Rb^+$ efflux, with an IC_{50} of 56 nM. Functionally both furosemide and its potent diuretic analog torasemide were mildly vasorelaxant in norepinephrine-contracted endothelium-denuded rat aorta, whereas PNU-37883A moderately increased basal tone and competitively inhibited P-1075 vasorelaxation with an IC_{50} of 0.4 μM. Thus, despite a sevenfold difference in functional potency, PNU-37883A blocked P-1075-mediated $^{86}Rb^+$ efflux and vasorelaxation in rat aorta independent of conventional cyanoguanidine K-ATP opener (i.e., [^{3}H]P-1075) and sulfonylurea K-ATP blocker

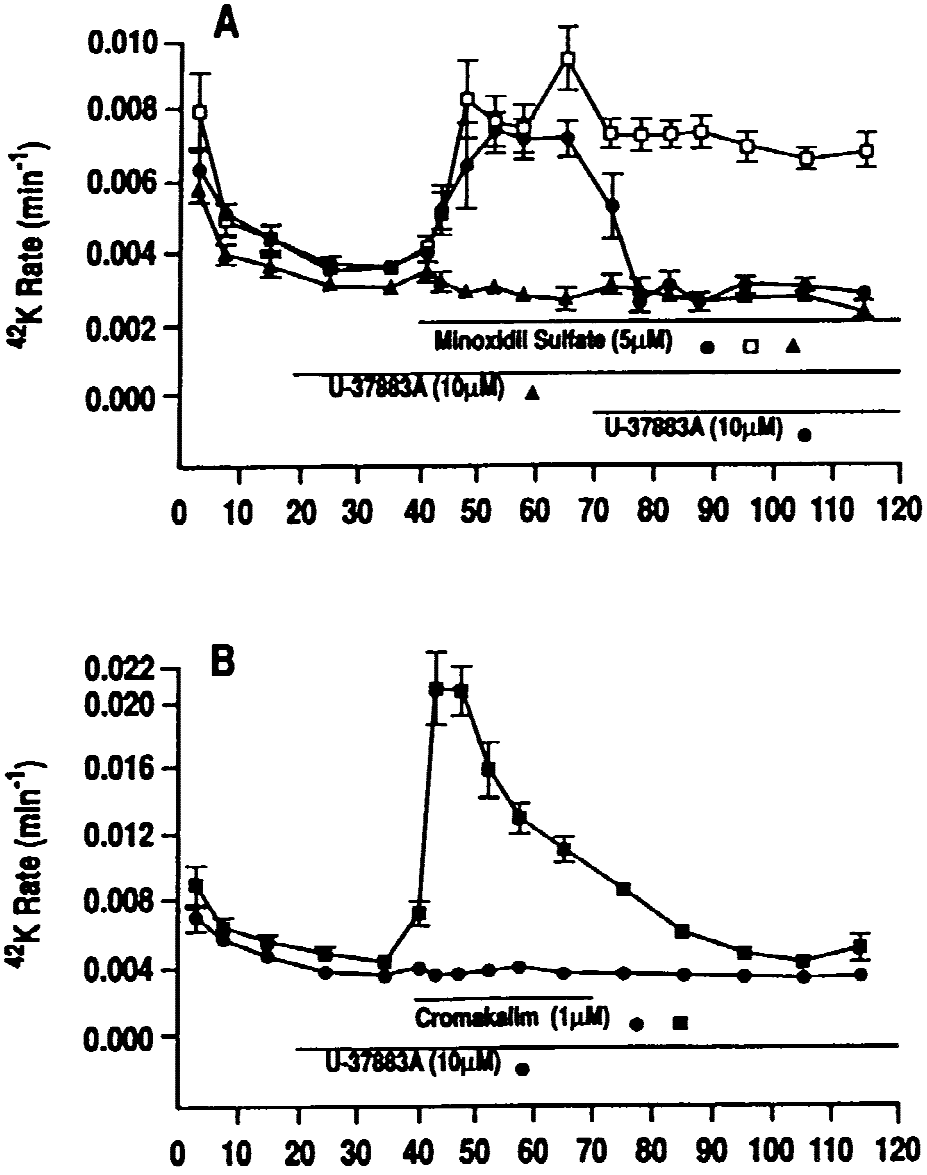

FIG. 4. Inhibitory effect of 10 μM PNU-37883A on minoxidil sulfate- and cromakalim-stimulated $^{42}K^+$ efflux from rabbit mesenteric artery. *A,* PNU-37883A inhibition of 5 μM minoxidil sulfate, which separately doubled basal $^{42}K^+$ efflux (*open squares*). PNU-37883A pretreatment significantly inhibited the effect of minoxidil sulfate (*closed triangles*), and 20-min PNU-37883A addition likewise rapidly blocked $^{42}K^+$ efflux (*closed circles*). *B,* PNU-37883A inhibition of 1 μM cromakalim, which separately induced a marked, transient fivefold $^{42}K^+$ efflux (*closed squares*). PNU-37883A pretreatment also significantly blocked the effect of cromakalim. Data are the mean ± S.E. from rabbit mesenteric artery rings obtained from five rabbits. From ref. 78, with permission.

([^{3}H]glyburide) binding sites. These findings further support the view that PNU-37883A's functional K-ATP blockade is mediated through a distinct vascular recognition site.

In Vivo K-ATP Channel Opener Reversal and Interactions

Meisheri et al. (78) and many others (16,27,28,54,69,97,99,108) have confirmed that, consistent with its *in vitro* profile, PNU-37883A antagonizes the hypotensive and vasodilatory effects of chemically different K-ATP channel openers in intact animals. In anesthetized cats, PNU-37883A (3.3 mg/kg i.v.) reversed the hypotensive and tachycardiac effects of minoxidil without affecting the pressor response to phenylephrine or the depressor responses to isoproterenol, nitroglycerin, or nitroprusside (78; Fig. 5). PNU-37883A (3 mg/kg i.v.) likewise prevented and reversed the fall in mean arterial pressure and rise in heart rate induced by pinacidil in anesthetized rats, independent of isoproterenol, nitroprusside, and nifedipine responses (78,108). As with the original finding with PNU-18177A (91), in conscious dogs the hypotensive and tachycardiac effects of minoxidil and cromakalim were reversed and prevented by PNU-37883A, given as a 3 mg/kg i.v. injection (78; Fig. 5) or sustained 6-μg/kg/min i.v. infusion (54). Similar K-ATP specificity was evident in anesthetized dogs, where PNU-37883A blocked cromakalim but not nitroglycerin, isoproterenol, serotonin, or 5-carboximidotryptamine (69).

ELECTROPHYSIOLOGICAL EVIDENCE OF K-ATP CHANNEL BLOCKADE

K-ATP Blockade and Specific Binding in *Xenopus* Oocyte Follicles

The first electrophysiological evidence that PNU-37883A blocks K-ATP channels was reported by Guillemare et al. (41), who tested the drug in follicle-enclosed *Xenopus* oocytes, a well-established model for vascular, bronchial, and central neuronal K-ATP channels (49). In this preparation 0.1 to 10 μM glyburide and PNU-37883A noncompetitively inhibited a 30 μM P-1060-activated outward K^+ current with IC_{50}s of 0.26 and 0.33 μM, respectively, as shown in Fig. 6. Qualitatively similar inhibitory effects were also seen with 10 μM PNU-37883A against K^+ currents activated by 300 μM cyclic adenosine monophosphate, 10 μM adenosine, and the paradoxical glyburide-activated K^+ current induced during dinitrophenol acidosis (42). Supporting these whole cell findings, in patch-clamped outside-out *Xenopus* oocyte membranes, a glyburide- and ATP-sensitive K^+ current (1.25 pA at 0 mV; 19 pS conductance) was reduced 76% by 10 μM PNU-37883A because of reduced open probability independent of channel conductance.

In further examining this K-ATP blockade, Guillemare et al. (41) also found low affinity, 62.5% specific [^{3}H]PNU-37883A binding in microsomal *Xenopus* oocyte follicular membranes, which could be displaced by unlabeled drug. Scatchard analysis identified a single binding site with a K_d of 454 nM and a B_{max} of 17.3 pmol/mg of protein. This specific [^{3}H]PNU-37883A binding appeared to be pharmacologically relevant since the active morpholinoguanidine analog PNU-52090 displaced the ligand, whereas the inactive analog PNU-42069D could not. These studies also offered evidence that PNU-37883A does not act through sulfonylurea receptor sites, since 10 μM glyburide could not displace [^{3}H]PNU-37883A. In line with this conclusion and the drug's euglycemic *in vivo* profile (73), PNU-37883A did not depolarize pancreatic RINm5F cells, did not block K-ATP channels from this cell line, and did not bind to RINm5F cell membranes (41; Fig.

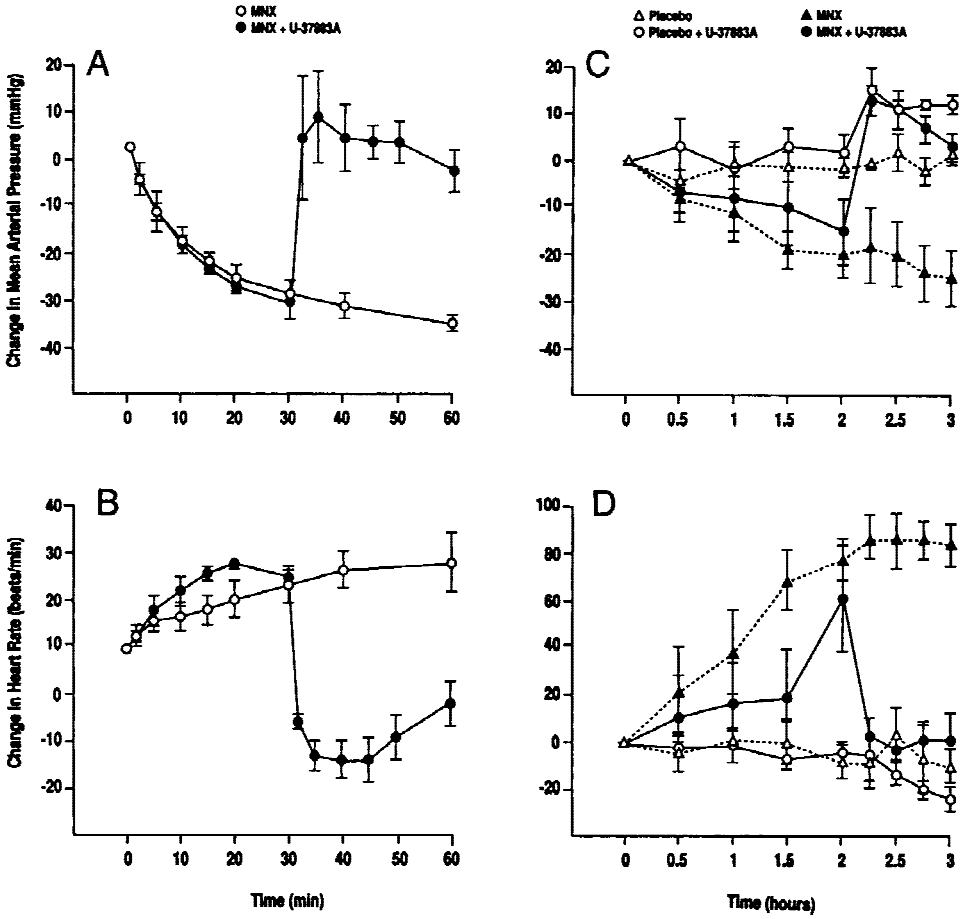

FIG. 5. PNU-37883A reversal of minoxidil (MNX)-induced hypotension and tachycardia in anesthetized cats (*A* and *B*, respectively) and conscious dogs (*C* and *D*). In cats, MNX (1 mg/kg i.v.) resulted in a significant fall in mean arterial pressure and a slight rise in heart rate (*open circles*), which were significantly antagonized by PNU-37883A (3.3 mg/kg i.v.) (*closed circles*). In dogs, PNU-37883A (3 mg/kg i.v.) alone slightly increased the mean arterial pressure and decreased the heart rate, while MNX (1 mg/kg p.o.) gradually reduced the mean arterial pressure and markedly increased the heart rate. Acute PNU-37883A 2 h after MNX rapidly and significantly normalized both parameters. Data are the mean ± S.E. for 4 animals/group. From ref. 78, with permission.

7). Thus, while exhibiting glyburide-like blocking effects in a model system equated with vascular K-ATP channels, PNU-37883A differed from glyburide in not affecting pancreatic beta K-ATP channels at pharmacological concentrations. These investigations thus provided the first electrophysiological evidence that PNU-37883A blocks vascular K-ATP channels with greater specificity than does glyburide.

Renal Tubular K-ATP Channel Blockade

PNU-37883A's functional and electrophysiological K-ATP blocking effects raised the question of whether its initially detected eukalemic diuresis might also be similarly me-

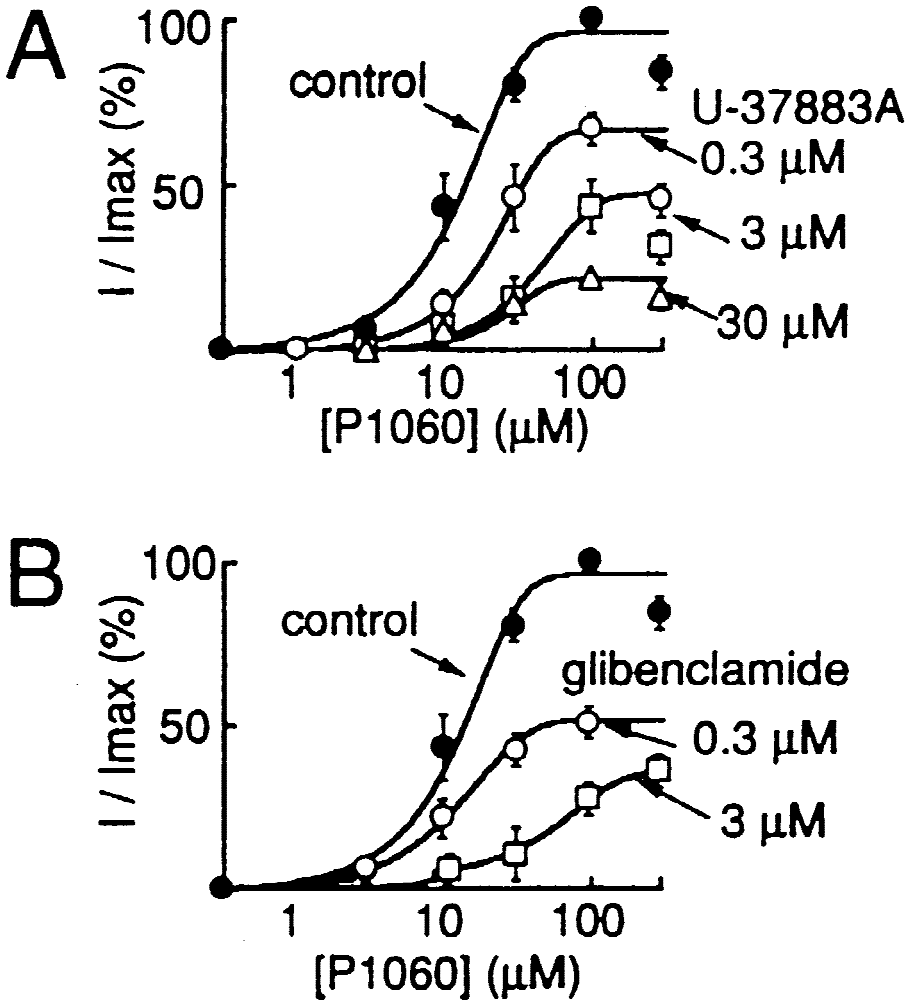

FIG. 6. Inhibition of P-1060-activated K^+ currents in follicle-enclosed *Xenopus* oocytes by PNU-37883A (*A*) and glyburide (glibenclamide; *B*). PNU-37883A or glyburide was added 5 min before the 2-min P-1060 application. I_{max} is the mean amplitude current activated by 100 μM P-1060 (control). Data are the mean ± S.E. from ≥8 oocytes from three frogs. From ref. 41, with permission.

diated. Since the drug's original synthesis, physiological studies had identified low-conductance ATP-sensitive, voltage-independent K^+ channels in the apical membrane of principal cells isolated from the thick ascending limb of rat loops of Henle, which appear to control membrane potential and the efficiency of the $Na^+/2Cl^-/K^+$ cotransporter pump (120). Wang et al. (119) and Schlatter (104) had also described a similar 30 pS ATP- and pH-sensitive K^+ channel in the apical membrane of rat cortical collecting duct that up-regulated during high K^+ intake, and that appeared responsible for K^+ secretion in this nephron segment. Indeed, based on this renal tubular physiology (118), Giebisch (35) in 1993 proposed that specific blockers of renal tubular K-ATP channels should enhance urinary Na^+ and volume excretion with far less K^+ loss than conventional diuretics. This

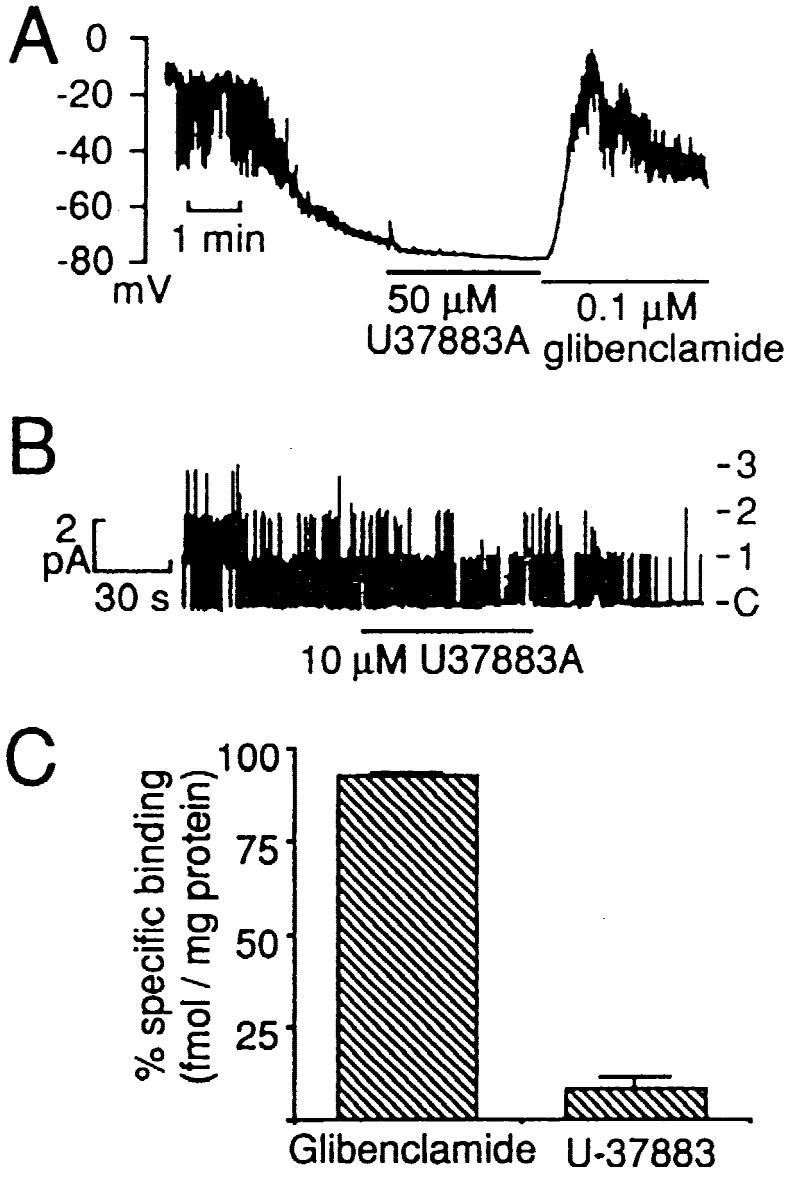

FIG. 7. Electrophysiological effects of PNU-37883A and glyburide (glibenclamide) in RINm5F cells. *A,* membrane potential of a RINm5F cell dialyzed with an ATP-free internal solution. Addition of 50 μM PNU-37883A had no effect on membrane potential, while 0.1 μM glyburide caused a dramatic depolarization. *B,* outside-out patch recording with an ATP-free internal medium, wherein K-ATP channel openings were not affected by 10 μM PNU-37883A. *C,* high-level specific binding of [^{3}H]-glyburide in RINm5F cell membranes, as compared with no significant binding by [^{3}H]PNU-37883A. From ref. 41, with permission.

speculation and the pharmacology of PNU-37883A thus prompted comparative evaluations between PNU-37883A and glyburide *in vivo* (72,73) and *in vitro* (116,117).

In anesthetized rats supporting their electrophysiological studies, Wang et al. (116) confirmed PNU-37883A's eukalemic diuresis (51,53,72,73,91), as doses of 1.8 to 15

mg/kg i.v. increased urinary volume and Na^+ excretion by up to 10-fold with only minor changes in the glomerular filtration rate and K^+ excretion (Fig. 8). PNU-37883A (15 mg/kg i.v. and 50 μM) did not affect electrolyte flux in microperfused rat proximal tubules, but 50 μM of drug significantly reduced volume, Na^+, and K^+ efflux out of microperfused loops of Henle by 37%, 44%, and 59%, respectively. Consistent with and possibly explaining this effect, in inside-out patch-clamped apical membranes isolated from thick ascending limb principal cells, 10 to 100 μM PNU-37883A blocked K-ATP channels with an IC_{50} of about 50 μM (Fig. 9). PNU-37883A (12.5 to 50 μM) also inhibited inside-out patch-clamped ATP-sensitive K channels in the apical membranes of cortical collecting duct principal cells, maximally reducing open probability 55% at depolarized potentials and suggesting a possible cytosolic site of action. PNU-37883A therefore blocked apical K-ATP channels in two nephron segments, but at concentrations about 10 to 100 times higher than those antagonizing vascular K-ATP channel openers in the rabbit mesenteric artery and blocking *Xenopus* oocyte follicular K-ATP channels.

On the basis of these findings, Wang et al. (116) concluded that PNU-37883A's tubular K-ATP blockade likely explains its eukalemic natriuresis by two actions. First, apical thick ascending limb K-ATP blockade would reduce the electrical driving force for Na^+ reabsorption and restrict the outward K^+ current normally feeding the $Na^+/2Cl^-/K^+$ cotransporter in this nephron segment. Such actions would bestow a relatively high natriuretic ceiling, albeit below that typically associated with conventional loop diuretics. Second, apical cortical collecting duct K-ATP blockade would restrict flow-dependent K^+ secretion in distal nephron segments, thus resulting in a relative K^+ sparing profile. Expanding on these observations, the eukalemic diuretic profile similarly reported for high-dose glyburide in rats (21) was also explored by Wang et al. (117). In these studies, 250 μM glyburide likewise reduced volume, Na^+, and K^+ efflux from microperfused loops of Henle by 34%, 53%, and 85%, respectively, and blocked apical thick ascending limb and cortical collecting duct K-ATP channels with IC_{50}s of ~150 μM. The fact that these chemically dissimilar agents exert qualitatively similar K-ATP channel blocking effects at two nephron sites offers strong evidence that this mechanism contributes to the eukalemic diuresis seen with both agents.

In Vitro Selectivity for Vascular K-ATP Channel Blockade

Two more recent studies have investigated PNU-37883A's selectivity for blocking vascular K-ATP channels. Higdon et al. (47) first compared PNU-37883A's K-ATP blocking effects in dog coronary artery and guinea pig myocardial cells. In patch-clamped and whole cell configurations, PNU-37883A blocked dog coronary artery K-ATP channels with an IC_{50} of 0.72 μM, while showing no effect on delayed rectifier or Ca^{2+}-activated K^+ currents. The drug also blocked P-1075-mediated vasorelaxation in U-46619-contracted dog coronary artery with an IC_{50} of 0.74 μM and similarly antagonized pinacidil and cromakalim. Conversely, in guinea pig myocytes, 30 μM PNU-37883A did not affect P-1075-induced action potential shortening, whereas glyburide was very effective at 0.1 μM. These tests demonstrate that PNU-37883A functionally antagonizes K-ATP channel openers in dog coronary and rabbit mesenteric arteries at drug concentrations akin to those blocking dog coronary K-ATP channels (i.e., IC_{50}s of 0.72 to 0.78

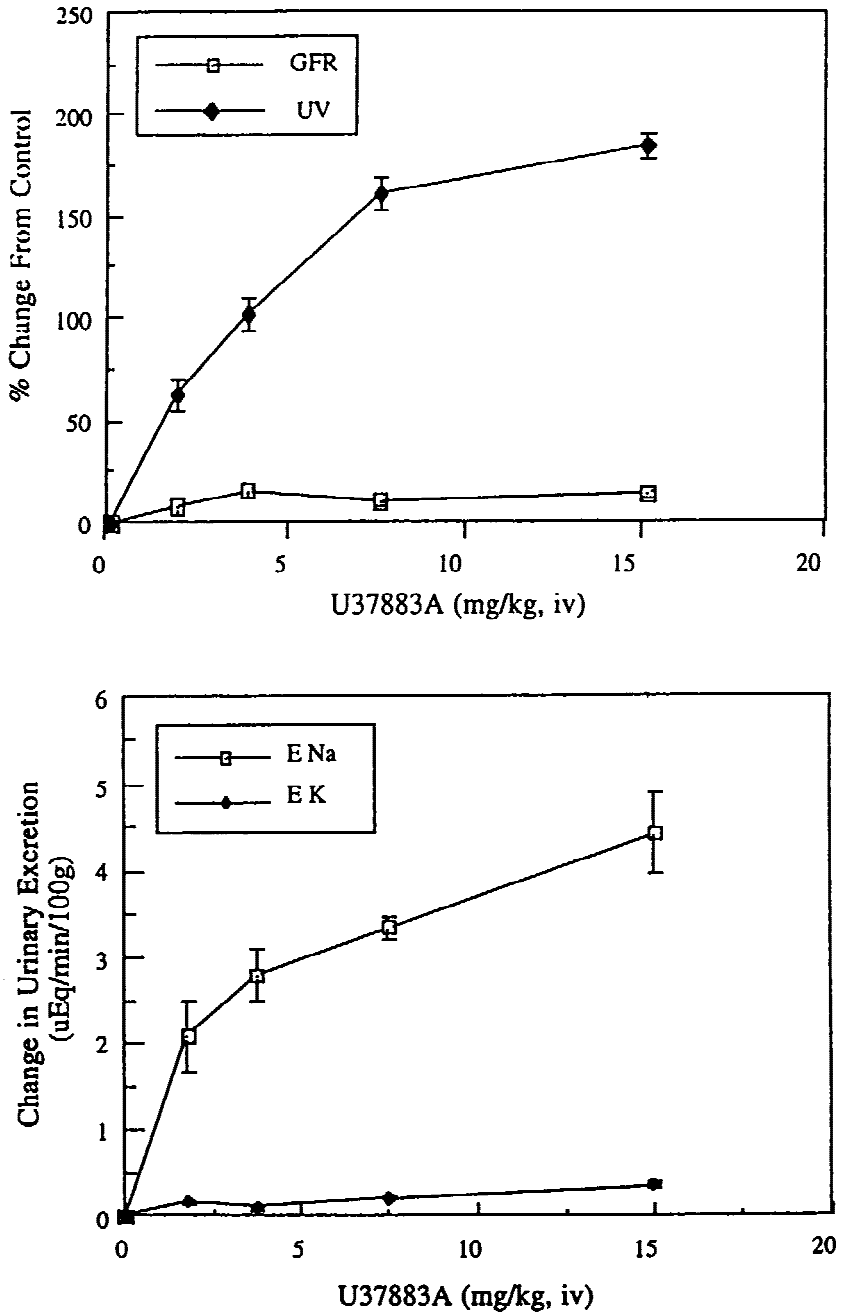

FIG. 8. Dose-dependent changes in renal function in anesthetized volume-expanded rats treated with i.v. PNU-37883A. *A*, percentage of changes in glomerular filtration rate (GFR) and urine flow rate (UV), calculated as treated − control ÷ control × 100% and expressed as the mean ± S.E. *B*, changes in urinary Na^+ and K^+ excretion (E) relative to saline-injected control rats, expressed as the mean ± S.E. PNU-37883A (1.5 to 15 mg/kg) resulted in marked significant increases in UV and Na^+ excretion independent of changes in GFR and kaliuresis. From ref. 116, with permission.

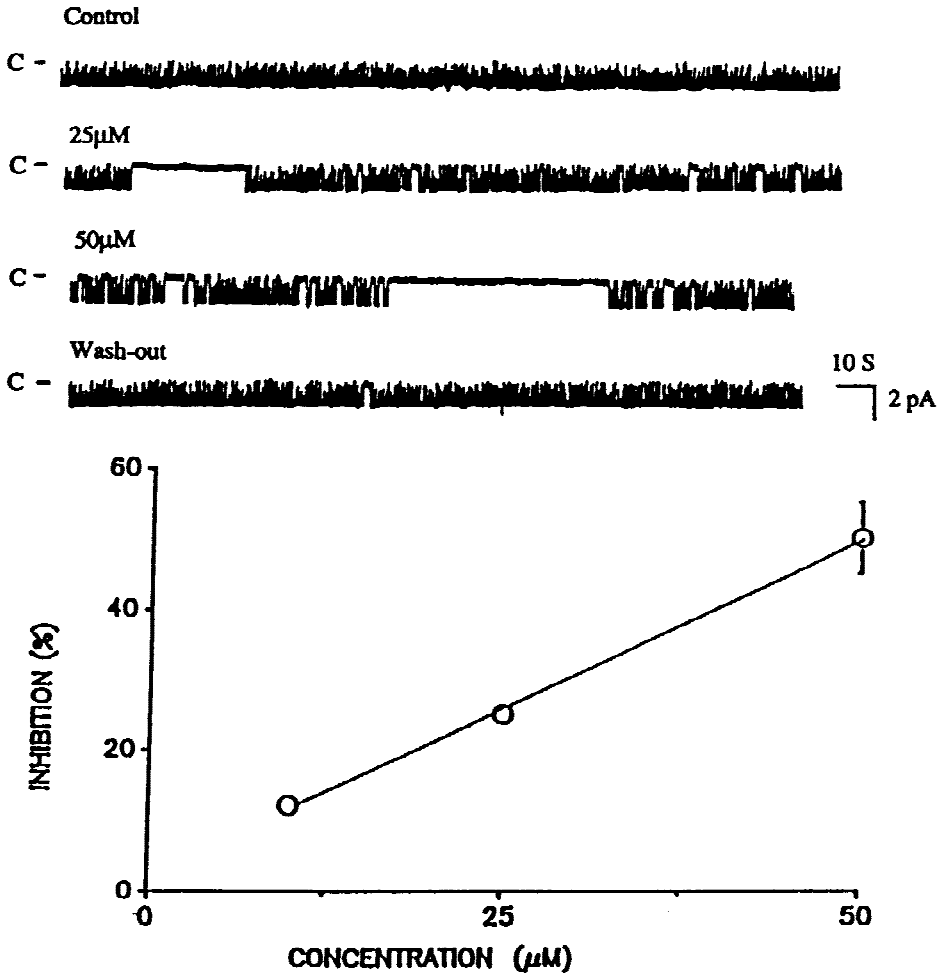

FIG. 9. PNU-37883A inhibition of an ATP-sensitive K^+ current in patch-clamped apical membranes from principal cells of rat cortical collecting duct. *Top,* representative traces showing the inhibition by 25 and 50 μM PNU-37883A (in the bath) and its washout. Pipette solution = 140 mM KCl, 1.8 mM $MgCl_2$, and 10 mM HEPES at pH 7.4; bath solution = 135 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES at pH 7.4. *Bottom,* PNU-37883A's concentration-response for K^+ channel inhibition. From ref. 116, with permission.

μM). In contrast, PNU-37883A does not antagonize K-ATP channel-mediated action potential duration shortening in guinea pig myocytes over this concentration range.

These findings have more recently been confirmed and expanded by Wellman et al. (122) in patch-clamped rat mesenteric artery, skeletal muscle (flexor digitorum brevis), and myocardial (right ventricular) cells. As in the rabbit mesenteric artery and rat aorta (47,71,76,78,87,88), 0.1 to 30 μM PNU-37883A significantly antagonized the vasorelaxant effects of 1 μM lemakalim in rat mesenteric artery cells with an IC_{50} of 1 μM. Under high K^+ and pinacidil-activated conditions, rat mesenteric artery cells displayed an inward ATP-dependent, glyburide-sensitive K^+ current, which was inhibited ~80% by 10 μM

PNU-37883A (IC_{50} = 3.5 μM; Fig. 10). Similar effects were seen under 0.1 mM ATP and cell dialysis conditions. The outward K-ATP current induced by 100 μM pinacidil at 0 mV was also inhibited 90% by 10 μM PNU-37883A. Conversely, the outward K-ATP currents induced in skeletal muscle and myocardial cells were unaffected by 10 to 100 μM PNU-37883A, but they were very effectively blocked by 10 μM glyburide. Maximal voltage-activated (K_v) whole cell K^+ currents in mesenteric cells were only marginally

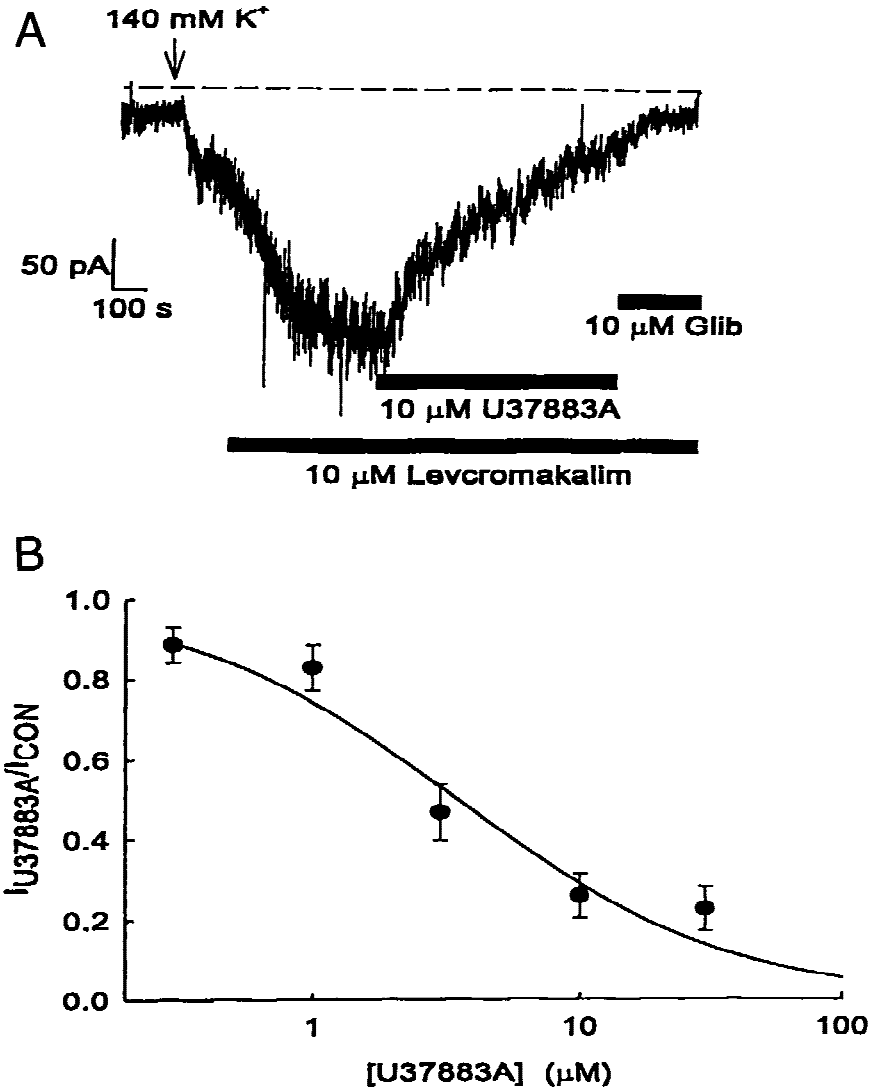

FIG. 10. PNU-37883A inhibition of a K-ATP current in rat mesenteric artery cells. *A*, representative trace showing 10 μM PNU-37883A blockade of 10 μM levcromakalim-stimulated K^+ current. Membrane potential = −60 mV; pipette solution containing 0.1 mM ATP and ADP; bath solution containing 140 mM K^+ with indicated drug levels. Glib, glibenclamide. *B*, PNU-37883A concentration-response curve for inhibiting 10 μM levcromakalim-stimulated K-ATP current (mean ± S.E.; 3 to 7 trials/conc.) with an IC_{50} of 3.5 μM. From ref. 122, with permission.

inhibited by 10 and 30 μM PNU-37883A, and these concentrations were likewise only weakly active against a Ba^{2+}-sensitive inward rectifier K^+ current (K_{ir}) in rat coronary artery cells. Collectively, these and the prior studies demonstrate PNU-37883A's selectivity for functionally antagonizing vascular K-ATP channel openers and blocking vascular K-ATP channels at ~1 μM, as compared with its relative inactivity against myocardial, skeletal muscle, and pancreatic beta K-ATP channels and vascular Kv and Kir channels. Twenty-five- to 50-fold higher levels are also necessary for its renal tubular K-ATP blockade.

Comparative *in Vivo* K-ATP Channel Blocking Selectivity

The above *in vitro* findings have been extended to intact animals by Ludens et al. (73), who compared PNU-37883A's diuretic, pinacidil blocking, and plasma glucose effects with those exerted by glyburide. In anesthetized rats, PNU-37883A and glyburide blocked pinacidil (0.2 mg/kg i.v.) by ~80% with parallel dose-response curves, but PNU-37883A was about 5 times more potent (ED_{50}s of 2 vs. 10 mg/kg; Fig. 11). Parallel natriuretic dose responses were also seen with both agents between 5.0 and 50 mg/kg i.v., but PNU-37883A again had greater efficacy and was about 9 times more potent than glyburide in evoking a net 0.6 mEq natriuresis (Fig. 11). Conversely, when equivalent pinacidil blocking doses of both agents were given to conscious nonfasted rats, PNU-37883A (15 mg/kg) was markedly natriuretic and minimally affected plasma glucose levels, while glyburide (25 mg/kg) was less natriuretic and cut plasma glucose levels nearly in half. These data thus support the *in vitro* findings that PNU-37883A is a more specific vascular K-ATP

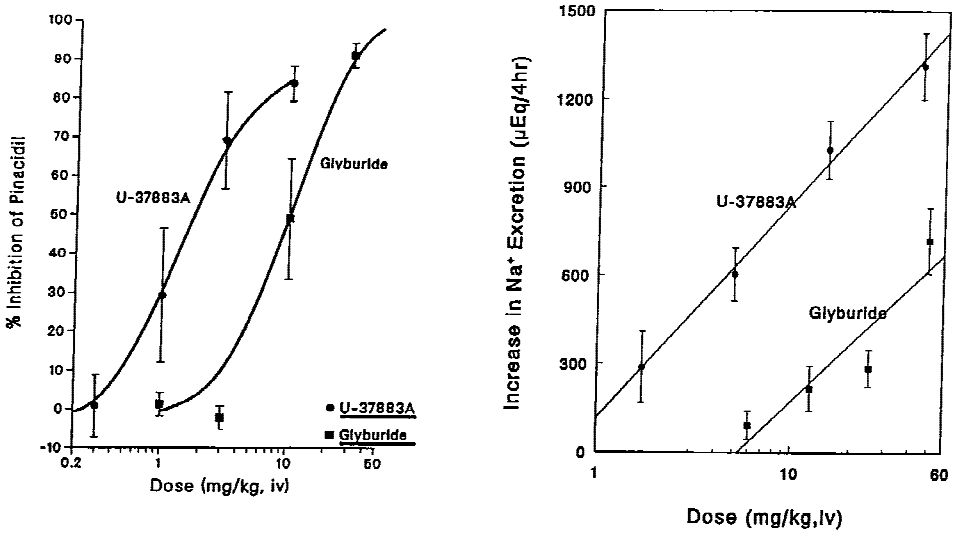

FIG. 11. Comparative pinacidil-blocking and natriuretic dose-responses for PNU-37883A and glyburide in rats. *A*, Blockade of the hypotensive effect of pinacidil (0.2 mg/kg i.v.) by i.v. PNU-37883A and glyburide in anesthetized rats, expressed as the percentage of inhibition relative to control rats (mean ± S.E.; $n = 3$/datapoint). *B*, net increase in 2-h urinary Na^+ excretion in conscious saline-loaded rats treated with i.v. PNU-37883A and glyburide, relative to control rats (mean ± S.E.; $n = 5$ or 6 rats/group). From ref. 73, with permission.

blocker than glyburide, and that PNU-37883A is also a more potent and efficacious diuretic.

RENAL PHARMACOLOGY

Diuretic Activity in Rats and Dogs

The diuretic effects of PNU-37883A and its analogs have been widely explored in rats and dogs prior to and subsequent to the discovery of their K-ATP channel blocking effects (21,22,51–53,72,73,76,91,116). In conscious rats, PNU-37883A (10 to 100 mg/kg p.o.) increased urinary volume and Na^+ exertion two- to sixfold without the kaliuresis seen with hydrochlorothiazide and furosemide or the K^+ retention common to amiloride and triamterene (91). A similar two- to 10-fold eukalemic natriuresis was also seen in conscious dogs with i.v. and p.o. PNU-37883A at up to 20 mg/kg (Fig. 12), but the drug was poorly tolerated at twice this dosage in both species because of myocardial depression. Ludens et al. (72) also compared PNU-37888A with equally natriuretic doses of furosemide and hydrochlorothiazide in conscious rats undergoing saline and water diuresis. During saline diuresis, PNU-37883A (1.7 to 15 mg/kg i.v.) maximally increased absolute and fractional Na^+ excretion sevenfold with little change in the glomerular filtration rate or plasma glucose, although the highest dose transiently reduced blood pressure and heart rate.

Early in its development, PNU-37883A's diuresis was also evaluated under high K^+ intake conditions (51). Conscious rats given potassium chloride p.o. to boost basal K^+ excretion threefold experienced further kaliuresis when treated with hydrochlorothiazide or furosemide, while rats treated with amiloride or triamterene experienced K^+ retention and hyperkalemia. Conversely, and consistent with its eukalemic profile, urinary K^+ excretion was generally unaffected by diuretic doses of PNU-37883A or PNU-18177A. Similar results were also seen in conscious dogs given potassium chloride, acetazolimide, or deoxycorticosterone to enhance baseline K^+ excretion 3- to 13-fold. Under these conditions, hydrochlorothiazide again potentiated K^+ excretion while PNU-18177A only slightly reduced it. Thus, prior to identifying PNU-37883A's possible diuretic mechanism, animal studies had shown that it negligibly affected urinary K^+ excretion when the tubular load of this cation was enhanced.

Suppression of Plasma Renin Activity

Also prior to the identification of PNU-37883A's K-ATP channel blockade, its effects on plasma renin activity were examined in conscious dogs (52). In hydropenic beagle dogs, PNU-37883A (6 to 60 mg/kg p.o.) resulted in a steep furosemide-like natriuresis distinguishing it from hydrochlorothiazide's lower efficacy and amiloride's K^+ retention. Further differing from these standards, PNU-37883A reduced plasma renin activity by 46 to 76% and failed to increase urinary aldosterone secretion. A threshold diuretic dose of PNU-37883A (1 mg/kg i.v.) also blunted the hyperreninemia induced by furosemide (1 mg/kg). Comparable 76% reductions in plasma renin activity were again seen in saline-loaded dogs given injections of PNU-37883A (10 mg/kg i.v.), but this dose increased blood pressure slightly and reduced the heart rate, such that reflex cardiovascular influences could not be excluded. To circumvent this concern, the drug was unilaterally infused into the renal artery (i.r.a.) of anesthetized dogs to measure its effect on renal venous

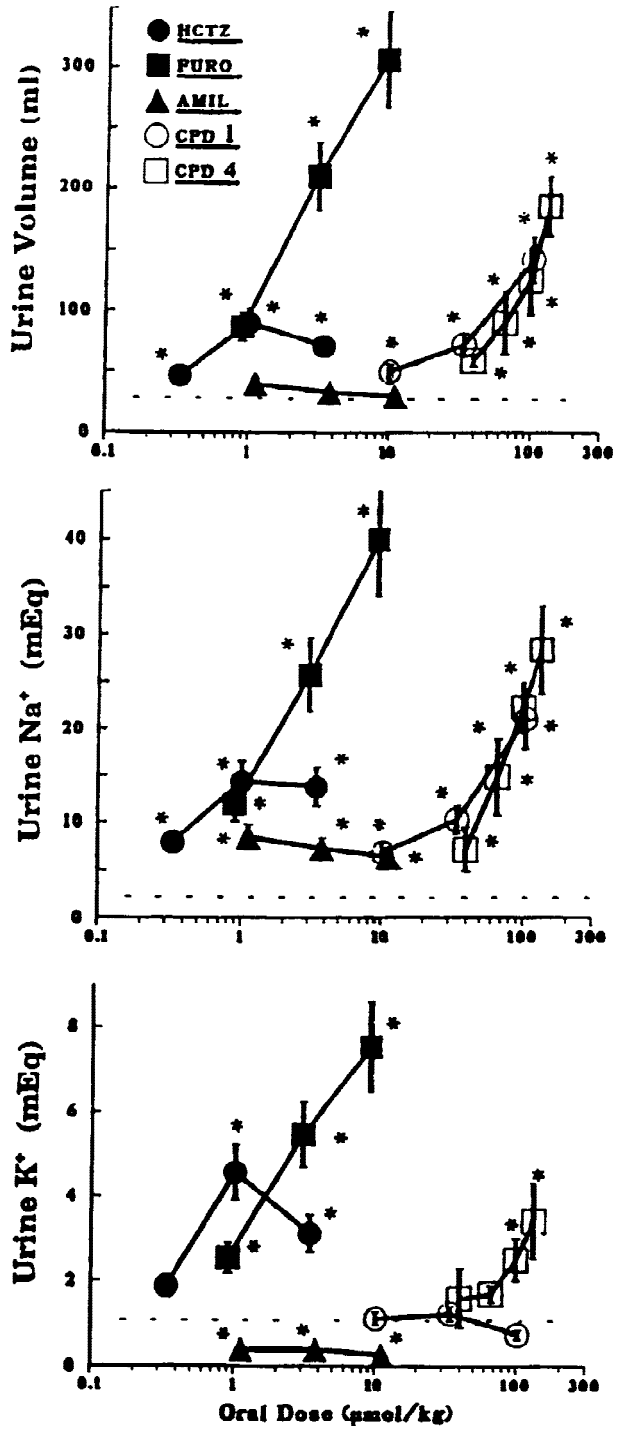

FIG. 12. Oral diuretic dose responses to the standard diuretics hydrochlorothiazide (HCTZ; *closed circles*), furosemide (FURO; *closed squares*), and amiloride (AMIL; *closed triangles*) as compared with PNU-18177A (CPD 1; *open circles*) and PNU-37883A (CPD 4; *open squares*). Datapoints show the mean 5-h urinary excretion from conscious female beagle dogs ($n = 6$/dosage) ± S.E. Doses shown as μM/kg and control excretion shown by dashed lines ($^*P \leq 0.05$ from control). Reprinted with permission from J. Med. Chem. 37:3693–3700. Copyright 1994. American Chemical Society.

plasma renin activity. In this model, PNU-37883A (1.5 and 3.0 mg/kg i.r.a.) reversibly suppressed renal venous plasma renin activity by 51% and 59%, respectively, and the suppression seen with the lower dose was independent of changes in urinary excretion, blood pressure, or heart rate, suggesting a selective reduction in renin release from juxtaglomerular cells. The subsequent discovery that PNU-37883A blocks K-ATP channels has led to the conclusion that the drug's suppression of plasma renin activity may be due to juxtaglomerular K-ATP blockade, membrane depolarization, and Ca^{2+} entry through voltage-sensitive channels. Such a mechanism would be consistent with previous findings with glyburide in mice and rats (68,92) and the established role of Ca^{2+} in regulating renin release (20,90).

Porcine Renal Cortical Binding

In an attempt to better understand its diuretic and the renal tubular K-ATP blocking effects, Meisheri et al. (76) examined [^{3}H]PNU-37883A binding in porcine kidney cortical microsomes. As with *Xenopus* oocyte follicular membranes, [^{3}H]PNU-37883A exhibited reversible, protein-dependent specific binding in this preparation with a K_d of 225 nmol and a B_{max} of 7.8 pmol/mg of protein. This binding was also sensitive to protease inhibitors and was not evident in rat insulinoma or whole brain membranes, where [^{3}H]glyburide shows high affinity binding. Pharmacological relevance was also demonstrated, as those diuretic PNU-37883A analogs capable of antagonizing K-ATP channel openers in rabbit mesenteric artery displaced tritiated drug, while those analogs devoid of such activities did not. It thus was concluded that PNU-37883A's low affinity binding to porcine kidney cortical membranes was specific, reversible, and tissue and protease sensitive, possibly representing a binding protein associated with the renal tubular K-ATP channel complex.

In Vivo Site and Mechanism of Diuresis

Studies of the *in vivo* site of diuretic action have also more recently been conducted with PNU-37883A in conscious rats (22,72). Under water diuresis conditions imposed to estimate fluid and Na^+ delivery out of the diluting nephron segment, furosemide (1.5 mg/kg) and PNU-37883A (15 mg/kg) increased fractional urine flow by 118% and 67%, respectively, and reduced renal papillary osmolality by 58% and 40%. It thus was concluded that PNU-37883A's eukalemic natriuresis was at least partially due to inhibited electrolyte transport in the thick ascending limb (72). In an extension of these studies, PNU-37883A (15 mg/kg) acutely increased urinary Na^+ excretion fivefold independent of changes in the glomerular filtration rate, but its efficacy was halved during water diuresis, presumably because of suppressed vasopressin release. This latter conclusion was confirmed when exogenous vasopressin substantially restored the drug's natriuretic efficacy. By comparison, furosemide (1.5 mg/kg) was consistently natriuretic under both conditions (22), thereby offering a clear contrast between PNU-37883A's apparent indirect inhibition of the apical $Na^+/2Cl^-/K^+$ cotransporter in the thick ascending limb, as opposed to furosemide's direct inhibition. These *in vivo* studies thus provided an important correlate to the

in vitro investigations of $Na^+/2Cl^-/K^+$ cotransporter and K-ATP function in this nephron segment (116) and the possible mode of PNU-37883A's diuretic action.

CARDIOVASCULAR AND MYOCARDIAL EFFECTS

In Vivo Cardiovascular Actions

PNU-37883A's cardiovascular effects have been examined in many different *in vivo* and *in vitro* models in conjunction with its early diuretic and more recent K-ATP blocking studies. In conscious and anesthetized rats, lower i.v. doses of PNU-37883A inducing mild diuresis and blocking exogenous K-ATP channel openers (≤5 mg/kg) minimally affected or slightly increased blood pressure, with modest decreases in heart rate (22,55, 72,73,108,113). Higher doses of PNU-37883A necessary for its marked diuresis (~15 mg/kg) acutely but transiently reduced blood pressure with a more pronounced, sustained bradycardia (22,55,72,73). Mild blood pressure increases and heart rate decreases were also seen in conscious dogs given a K-ATP blocking 6-h PNU-37883A infusion (2.2 mg/kg i.v.; 54), whereas a more natriuretic dose of 10 mg/kg transiently increased blood pressure and reduced heart rate (52). Compared with these species, in anesthetized cats K-ATP blocking doses of PNU-37883A (≤5 mg/kg) minimally affected basal blood pressure, heart rate, or vascular resistance (16–19,27,28,78,99).

Hemodynamic and Myocardial Effects in Anesthetized Rats and Dogs

Based on the cardiac toxicity seen during its early diuretic development, PNU-37883A's acute cardiovascular and myocardial effects were monitored in anesthetized dogs given stepped i.v. doses of 0.5 to 32 mg/kg (55). Lower doses (≤8 mg/kg) progressively increased mean arterial and left ventricular systolic pressures with few heart rate changes, while higher doses fatally reduced all three parameters in three of four dogs at 16 to 32 mg/kg. The increases in left ventricular monophasic effective refractory period and action potential duration and decreases in left ventricular contractility contributing to these deaths were evident over the entire dose range, but did not become significant until cumulative doses of 2 to 8 mg/kg had been achieved. As per these functional parameters, electrocardiographic changes were minimal at ≤8 mg/kg, but PR, QRS, and QTc intervals were prolonged at ≥16 mg/kg independent of cardiac rhythm. Very similar progressive reductions in mean and phasic arterial pressure, heart rate, and left ventricular contractility were also seen in anesthetized rats given stepped i.v. PNU-37883A, which became most pronounced at ≥10 mg/kg (acute i.v. $LD_{50} \cong 50$ mg/kg; Fig. 13).

In Vitro Myocardial Effects

PNU-37883A and selected analogs were tested for their direct myocardial effects in Langendorff perfused rat hearts in the early 1970s. As subsequently reported in 1995 (53), stepped 0.03 to 3 μg of PNU-37883A injected into the coronary perfusate resulted in progressive sustained reductions in left ventricular contractility (−5 to −73%) and rate (−5 to −95%). Lacking specific ion channel modulators with which to probe the drugs' diuretic and cardiotoxic effects, work on this series ended in 1975 and was not resumed until PNU-37883A was confirmed to be a selective antagonist of vascular K-ATP channel

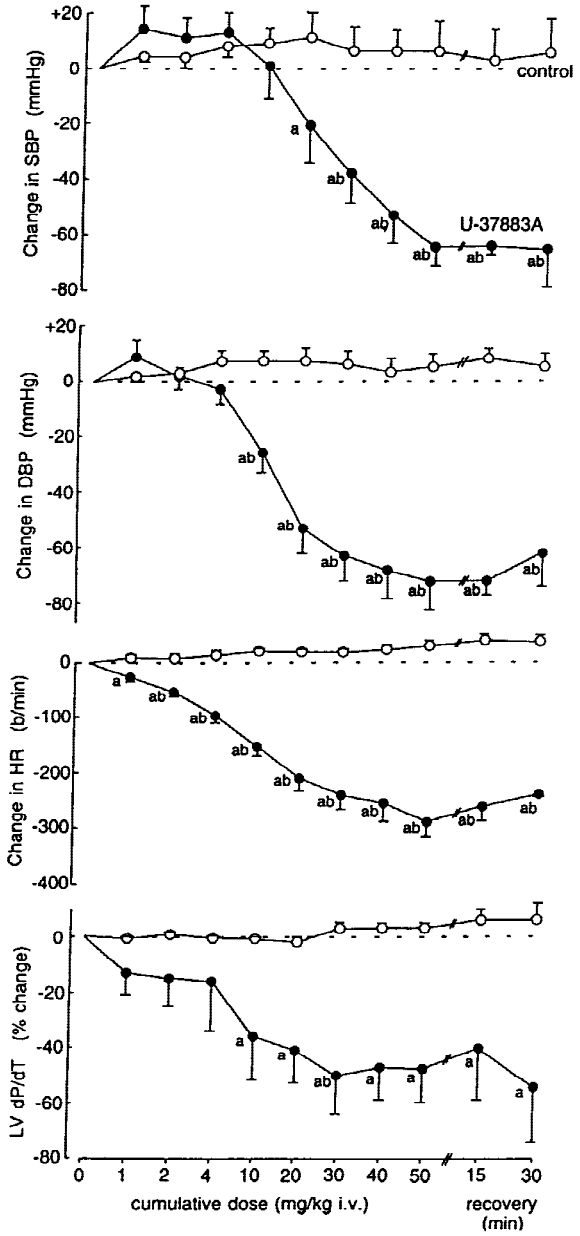

FIG. 13. Cardiovascular effects of i.v. PNU-37883A in chloralose-urethane-pentobarbital anesthetized rats. Shown are the mean changes (±S.E.) in systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and left ventricular contractility (LV dP/dT) seen with cumulative PNU-37883A (1 to 50 mg/kg) injected over 35 min. *Open circles*, control; *closed circles*, PNU-37883A ($n = 4$). *a*, $p \leq 0.05$ from control; *b*, $p \leq 0.05$ from pretreatment (0 time). From ref. 55, with permission.

openers in the early 1990s (78), which led to a comparison between PNU-37883A and glyburide in oxygenated superfused rabbit ventricular muscles (55). In this preparation, 0.1 to 10 μM PNU-37883A minimally affected the force of contraction or effective refractory period, but 50 μM PNU-37883A reduced the contractility (−33% at 3 Hz), increased the effective refractory period (28% at 3 Hz), and markedly increased the conduction time (109% to 244% at 1 and 3 Hz). Conversely, glyburide did not affect any parameter at 0.1 to 10 μM and only marginally prolonged conduction time at 50 μM.

The specific mechanisms responsible for PNU-37883A's myocardial depressant effects in intact animals, perfused hearts, and superfused ventricular strips remain speculative, but the available evidence seems to discount a direct relationship with myocardial K-ATP blockade. This conclusion is based on the observations that, while glyburide and PNU-37883A potently block vascular K-ATP channels (47,122), glyburide more potently blocks myocardial K-ATP channels (122) without the marked cardiodepressant effects seen with PNU-37883A (55). K-channel-independent cardiodepression was also seen in anesthetized dogs, as graded i.v. PNU-37883A reduced left ventricular contractility prior to increasing action potential duration (55). Consistent with these findings, myocardial (sarcolemmal) K-ATP channels are regarded to be closed under normal metabolic conditions (84) and thereby relatively insensitive to specific K-ATP blockers such as glyburide (23,25), whereas nonspecific K-channel blockade by tetraethylammonium, 3,4-diaminopyridine, and barium increases *in vitro* myocardial contractility (123). Additionally, the selective vascular K-ATP channel openers pinacidil and cromakalim (85,107, 110) reduce myocardial contractility at supra-vasorelaxant concentrations (23,103,123), and this negative inotropy is attenuated by glyburide (82,103). These data and reports thus strongly suggest that the generalized cardiodepression seen with higher doses and concentrations of PNU-37883A cannot be solely and directly attributed to its blockade of cardiac sarcolemmal K-ATP channels.

It must also be further emphasized, however, that PNU-37883A's vascular and newly documented intracellular K-ATP blocking effects could indirectly precipitate or contribute to its cardiac toxicity, as scrutinized from three perspectives. First, analog PNU-18177A reduced baseline coronary blood flow in anesthetized dogs (M. G. Wendling, unpublished data) and blocked minoxidil's coronary vasodilation in conscious dogs (91). Since high-dose glyburide has also been shown to reduce basal coronary perfusion (9,57,98), reactive (8) and β_1 adrenoceptor-mediated coronary vasodilation (83), and coronary microvascular vasomotion (63), it is likely that high-dose PNU-37883A has qualitatively similar effects. Second, overwhelming evidence indicates that myocardial K-ATP channels play a crucial role in protecting the heart from ischemic damage (5,37,64). More specifically, brief repetitive episodes of coronary artery occlusion (89,124,125) or the acute administration of K-ATP channel openers (7,40,100) appears to activate myocardial K-ATP channels located in the inner mitochondrial membrane (33,34,50,58,70), which, possibly thorough a protein kinase C (102,121) or actin cytoskeleton pathway (10), protect the heart from subsequently more severe ischemia. Confirming that these cardioprotective effects are K-ATP mediated, activation of a myocardial mitochondrial K^+ uniport has been associated with biochemical changes regarded as protective against ischemia (48), and these channels (59) and their associated myocardial protective effects are blocked by glyburide and 5-hydroxydecanoate (6,38,39,101). Finally, as described in the ensuing section, moderate concentrations of PNU-37883A block a putative K-ATP current in mitochondria (111)

and phosphatidylserine synthesis in endoplasmic reticulum (74) isolated from rat hepatocytes, effects which could affect mitochondrial membrane potential, metabolism, and Ca^{2+} homeostasis. It is therefore possible that a significant portion of PNU-37883A's cardiodepression and acute cardiotoxicity may be related to either its blockade of coronary artery K-ATP channels (thereby restricting basal or demand-dependent increases in myocardial perfusion) and/or its blockade of cardioprotective mitochondrial K-ATP channels. Based on this speculation, future studies combining PNU-37883A with a standard or relatively cardioselective K-ATP channel opener such as BMS 180448 or BMS 191095 (5,29,95) could help to determine whether and to what extent PNU-37883A's toxicity is influenced by these K-ATP channel blocking effects.

INTRACELLULAR CHANNEL AND MEMBRANE EFFECTS

As noted above, two publications have reported that PNU-37883A has intracellular channel blocking, specific binding, and membrane permeability effects. Szewczyk et al. (111) examined the effects of PNU-37883A on rat hepatic mitochondrial microsomes using the Ca^{2+} ionophore A-23187 to induce mitochondrial swelling and K^+ influx through putative K-ATP channels. The resultant K^+ influx was concentration dependently inhibited by PNU-37883A, with an IC_{50} of 89 μM independent of membrane potential, and was unaffected by the non-K-ATP blocking analog PNU-42069D. As in other membrane and cellular preparations, PNU-37883A appeared to act at a different regulatory site than did glyburide. Thus, PNU-37883A also has K^+ current blocking effects in rat hepatic mitochondrial membranes, but at concentrations substantially higher than those blocking vascular K-ATP channels.

On the basis of these and prior findings with other K-channel blockers, Makowski et al. (74) also profiled the effects of PNU-37883A on phosphatidylserine synthesis in rat liver endoplasmic reticulum membranes. With a phospholipid base exchange reaction, PNU-37883A concentration dependently enhanced phosphatidylserine synthesis by up to 2.5-fold (EC_{50} = 54 μM), while PNU-42069D and glyburide were inactive. Further tests with modulators of cation transport and Ca^{2+}-ATPase indicated that PNU-37883A's phosphatidylserine stimulatory effect was Mg-ATP and Ca^{2+}-dependent and sensitive to the Ca^{2+} ionophore A-23187 and the Ca^{2+}-ATPase inhibitor thapsigargin. This effect was also associated with low affinity [^{3}H]PNU-37883A binding (K_d = 9 μM) and enhanced Ca^{2+} uptake. On the basis of this profile, it was concluded that PNU-37883A does not directly affect endoplasmic reticulum integrity, K^+ permeability, membrane potential, or the K_m of the base exchange enzyme. However, PNU-37883A does seem to indirectly activate phosphatidylserine synthesis by enhancing Ca^{2+} entry and potentiating the exchange reaction's sensitivity for Ca^{2+}. As noted earlier, using these techniques with intramyocardial membrane preparations could aid in the understanding of the cardiac depressant effects of high doses and concentrations of PNU-37883A.

CENTRAL NERVOUS SYSTEM EFFECTS

In Vitro Neuronal Effects

Only limited neuronal data have been reported with PNU-37883A. Lin et al. (67) have recently shown that PNU-37883A blocks K-ATP channels in neurons isolated from rat

caudate-putamen. When activated by the dopamine D_2 receptor agonist quinpirole (10 μmol), these cells exhibit an 85 pS K^+ current that is sensitive to 1 to 10 μM glyburide. Very low concentrations of PNU-37883A (0.1 to 1 μM) also inhibited this K^+ current with an IC_{50} of ~0.1 μM, because of reduced open probability independent of changes in channel conductance or reversal potential. PNU-37883A thus proved to be about 50 times more potent than glyburide and distinguished these neuronal K-ATP channels from those in vascular smooth muscle and pancreatic beta cells where glyburide is far more potent. This difference in K-ATP channel sensitivity between PNU-37883A and glyburide could prove very important in further exploring the electrophysiology and pharmacology of these and other central neuronal K-ATP channels.

Effects on Efferent Sympathetic Nerve Activity

Inferior cardiac efferent sympathetic nerve activity has been recorded in studies of baroreceptor intact anesthetized cats treated with minoxidil and PNU-37883A (Humphrey and McCall, unpublished data). The −30 mmHg hypotension achieved with minoxidil (1 mg/kg) was associated with a 115% increase in inferior cardiac sympathetic nerve activity, likely due to the withdrawal of the sympathoinhibitory baroreceptor tone. The addition of PNU-37883A (3.3 mg/kg i.v.) at this point normalized blood pressure and sympathetic nerve activity, thereby suggesting that this agent does not directly affect baroreceptor function or spontaneous inferior cardiac sympathetic tone.

COMPARISON K-ATP CHANNEL BLOCKERS

Glyburide

A detailed comparison between PNU-37883A and other known K^+ channel blockers is beyond the scope of this review, but other sections of this review have characterized PNU-37883A's pharmacology relative to that of the sulfonylurea glyburide. This agent is clearly more potent than PNU-37883A in blocking cardiac (47,112), skeletal muscle (112), and pancreatic beta K-ATP channels (41), is equipotent in blocking vascular (47,112) and *Xenopus* oocyte follicular K-ATP channels (41), but is less potent in blocking renal tubular (116,117) and quinpirole-activated neural K-ATP currents (67). Functionally glyburide differs in being hypoglycemic (73) and more potent than PNU-37883A in antagonizing K-ATP channel openers in isolated blood vessels (71,78,87), but it is less potent in reversing K-ATP opener-mediated hypotension (73), blocking postischemic hyperemia (80), inducing natriuresis (21,72,73,116,117), and depressing myocardial function (55).

Tedisamil

Besides glyburide, three other known K-ATP blockers deserve mention because of their structures and pharmacological profiles. The dialkyl diazabicyclo[3.3.1]nonane tedisamil (KC 8857; Fig. 1) resembles PNU-18177A in having bilateral symmetry and paired cycloalkyl groups and, like PNU-37883A, the drug inhibits cromakalim- and minoxidil sulfate-induced $^{86}Rb^+$ and $^{42}K^+$ efflux from rat aorta (0.01 to 1 μM; 12). This agent is also less potent than glyburide in preventing cromakalim vasorelaxation in rat aorta. With

similar findings, Kreye et al. (65) concluded that tedisamil is a noncompetitive K-ATP blocker that may also activate voltage-dependent Ca^{2+} channels. Like PNU-37883A, tedisamil in rats increased blood pressure, reduced heart rate, and prolonged QRS and QT intervals (44), but it may have much stronger antiarrhythmic effects against post-myocardial infarction ventricular arrhythmias (115). Thus, tedisamil's vascular and low-dose hemodynamic effects are common to PNU-37883A, but it differs in having more pronounced class III antiarrhythmic effects and slightly different toxicity (i.e., respiratory depression and ventricular arrhythmias; 44).

ZM181,037

Kau et al. (61) have described ZM181,037 (formerly ICI-181,037; Fig. 1) as a novel eukalemic diuretic with thiazide-like efficacy that does not affect urinary K^+ excretion or plasma K^+ levels. Despite its diuretic action, ZM181,037 failed to reduce blood pressure in hypertensive rats (62). Subsequent vascular studies demonstrated that like PNU-37883A, this agent also blocks cromakalim in 20 mM KCl-contracted rat aorta with a pK_b of 6.5, and similar K-ATP opener antagonism was seen in guinea pig portal vein and detrusor muscle. ZM181,037 also increased spontaneous myogenic contractions in these later tissues and prevented cromakalim-induced $^{86}Rb^+$ efflux akin to PNU-37883A and glyburide. Also like PNU-37883A but unlike glyburide, ZM181,037 did not reduce plasma glucose levels in dogs. ZM181,037 thus resembles PNU-37883A in exerting an eukalemic diuresis in two species, blocking vascular K-ATP channel openers, and not inducing insulin-dependent hypoglycemia.

Imidazol(id)ines

Certain imidazolines and imidazolidines such as phentolamine and alinidine (Fig. 1), respectively, have also been shown to block vasorelaxant K-ATP channel openers in dog and rat arteries (75), and phentolamine stimulates insulin release from superfused mouse pancreatic beta cells (60). Such agents also have affinity for widely distributed imidazoline binding sites (81), which resemble PNU-37883A's binding in being distinct from sulfonylurea sites (13). Alinidine also has bradycardiac and kaliuretic diuretic effects in conscious rats (unpublished observations), suggesting that it may have cardiac and/or renal tubular effects. Further comparisons among PNU-37883A, glyburide, ZM181,037, and selected imidazolines such as alinidine could thus prove very helpful in defining the structural and functional differences among these various K-ATP channels.

K-ATP REGULATION AND POSSIBLE MECHANISMS OF PNU-37883A BLOCKADE

The mechanism by which PNU-37883A blocks vascular, renal tubular, and neuronal K-ATP channels has not been defined. Indirect evidence suggests that PNU-37883A may act on a plasmalemmal protein associated with the K_{IR} pore or the tetrameric sulfonylurea binding proteins comprising the K-ATP channel complex (1,30). This speculation is indirectly based on PNU-37883A's relative vascular selectivity (41,47,122), its synergistic K-ATP blocking effects when combined with glyburide (87), and its binding to non-sulfonylurea sites in *Xenopus* oocyte follicular, vascular, porcine renal cortical, and in-

tracellular membranes (41,67,74,76). Such a mechanism thus may represent the functional antithesis of the model recently described by Hambrock et al. (43) and Schwanstecher et al. (105), whereby the sulfonylurea 2B protein of the K-ATP complex acts as a "receptor" for diverse K-ATP channel openers. As such, PNU-37883A's binding or membrane interaction may result in conformational changes in the central K_{IR} pore, which then restricts K^+ efflux.

Moving beyond this paradigm, it is possible that PNU-37883A's K-ATP blockade also involves some direct or indirect influence on the inner cell membrane, since its effect on apical K-ATP channels isolated from rat cortical collecting ducts was enhanced at lower pH (116). PNU-37883A is normally protonated at physiological pH, suggesting that its improved K-ATP blockade under more acidic circumstances may be related to membrane protonation and/or other cytosolic interaction. Such a mechanism has recently been discussed by Deutsch et al. (26), who proposed that varied ATP binding and charge density on the inner sarcolemmal membrane affect its interaction with the channel pore and overall ATP sensitivity. In studies closely related to this question, Shyng and Nichols (106) and Baukrowitz et al. (11) have more recently explored the role of intracellular phosphatidylinositol phosphates (PIPs) in patch-clamped K-ATP channels ($K_{IR}6.2$ with the type 1 sulfonylurea receptor) expressed in COSm6 cells. These researchers found that the "rundown" of excised patches could be prevented by adding PIP_2, which increased K_{ir} open probability and the K^+ current and which was sensitive to polylysine, Ca^{2+}, or spermine. These findings suggest that PIPs bind to positive charges on the intracellular membrane and allosterically interact with the carboxyl terminus of K_{IR} of 6.2 to prevent the inhibitory effects of ATP. This may explain the progressive increase in ATP sensitivity seen in patches sequentially excised from PIP_2-treated cell membranes. It may also explain the wide-ranging ATP sensitivities seen in native channels from different tissues and cell types (2). Further studies are thus needed to link tissue PIP_2 levels to ATP sensitivity, to establish whether such modulation occurs functionally, and to determine whether PNU-37883A might affect channel ATP sensitivity through this mechanism.

PNU-37883A AS A RESEARCH TOOL

Vascular Smooth Muscle

Because of its potency, solubility, and relative selectivity, PNU-37883A has been increasingly used as a K-ATP blocker to establish whether endogenous and exogenous agents relax vascular smooth muscle by this mechanism. These studies have commonly included a standard K-ATP opener (15–19,27,28,45,54,97,99,108,112) or transient ischemia (80; Fig. 14) to reduce perfusion pressure or vascular tone. In each instance, PNU-37883A has consistently antagonized many different K-ATP channel openers and hyperemic responses at relatively low *in vivo* doses ($\leq$5 mg/kg) and *in vitro* concentrations ($\leq$10 μM). Representative of such studies, that by Heaton et al. (45) reported that PNU-37883A did not modify the vasodilation caused by human adrenomedullin or calcitonin gene-related peptide in rat pulmonary artery, suggesting that these substances probably are not K-ATP openers in this vascular bed. Likewise in rat hindquarters, bradykinin (86) and the endorphins endomorphin-1, [N-MePhe^3, D-Pro^4]-morphoceptin, [D-Ala^2, MePhe^4, gly (ol)^5] enkephalin, and nociceptin (16,18) were unaffected by PNU-37883A, as was hypoxic venodilation (66).

In cats, PNU-37883A failed to antagonize des-Arg^9-bradykinin (27) and adenosine (19)

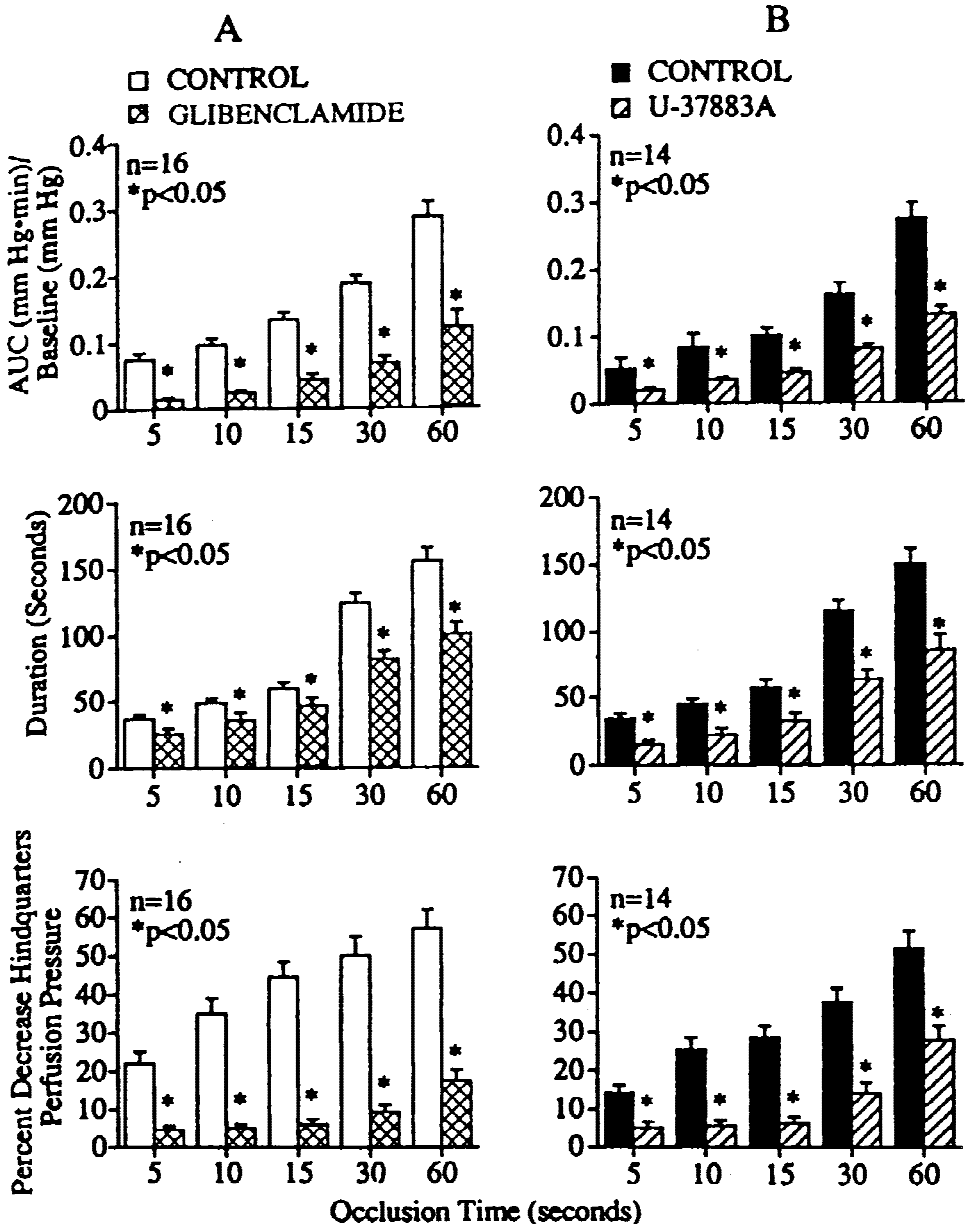

FIG. 14. Effects of glyburide (glibenclamide: *A*) (20 mg/kg i.v.) and PNU-37883A (*B*) (5 mg/kg i.v.) on the reactive hyperemic response in cat hindquarters. *Graphs* depict the area under the curve (AUC), duration, and percentage of decrease in hindquarters perfusion pressure evoked by 5- to 60-s arterial inflow occlusions under control conditions and 10 min after glyburide (*crosshatched bars*) or PNU-37883A (*hatched bars*). Significant attenuation to each measure of reactive hyperemia was seen with both K-ATP channel blockers (*$P \leq 0.05$ from control; $n = 14$ to 16 cats/group). From ref. 80, with permission.

in the perfused pulmonary artery; human proadrenomedullin, adrenomedullin (16), T-kinin, bradykinin, acetylcholine, and albuterol (18) in the perfused mesenteric artery; or kallidin, des-Arg^9-bradykinin, des-Arg^{10}-kallidin (99), diamaprit, *R*-(-α)-methylhistamine, bradykinin, and acetylcholine (17) in the perfused hindquarters. The most detailed investigations in this species have been by DeWitt et al. (28), who examined the effects of PNU-37883A and glyburide on the responses to many different vasoactive agents under normal and U-46619-vasoconstricted conditions (Fig. 15). In perfused cat hindlimbs, 5 mg/kg of PNU-37883A and glyburide per kg uniformly blocked the vasorelaxant effects of lemakalim, independent of basal perfusion pressure or U-46619. Contrasting interactions were seen in the perfused cat pulmonary artery, however, as PNU-37883A potentiated the pressor responses to U-46619, PGD_2, $PGF_{2\alpha}$, and BAY K 8644, suggesting that PNU-37883A-sensitive K-ATP channels in this tissue normally buffer such vasoconstrictors. Conversely, under same conditions glyburide blunted the vasoconstrictor effects of U-46619, PGD_2, and $PGF_{2\alpha}$. This differential response was not evident during U-46619-induced pulmonary constriction, as both agents antagonized known K-ATP openers, but not vasodilators acting through alternative pathways (i.e., PGE_1, isoproterenol, or felodipine). Thus, while confirming the K-ATP blocking effects of PNU-37883A and glyburide in the cat hindlimb and pulmonary artery, these researchers also identified several key differences in their pulmonary vascular interactions that may offer clues as to how K-ATP channels help to regulate smooth muscle tone in this organ.

Equally incisive studies have utilized PNU-37883A as a selective K-ATP channel blocker in dogs, as typified by the finding that the cutaneous vasodilation resulting from

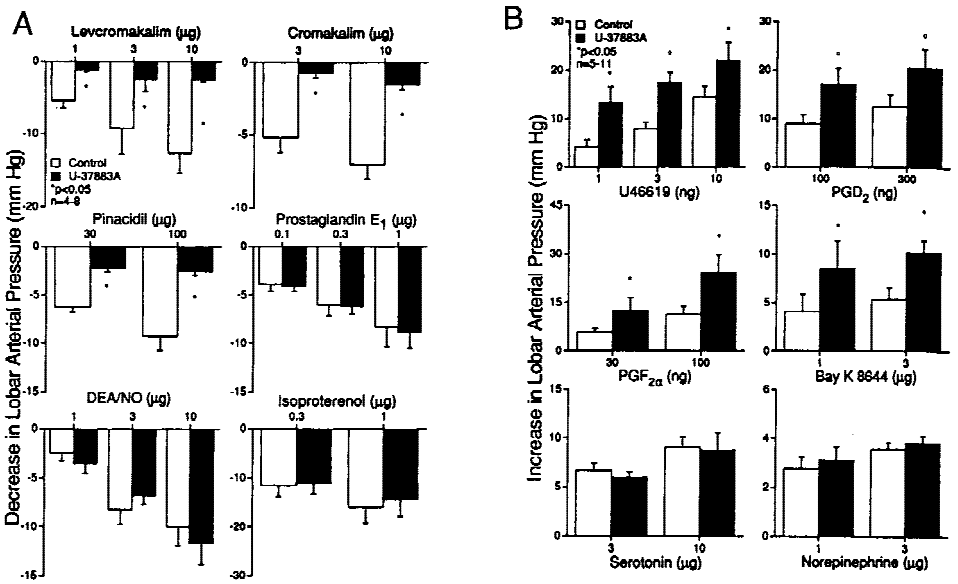

FIG. 15. Effects of PNU-37883A on pulmonary artery responses to depressor and pressor agents in anesthetized cats. Shown are the mean decreases ± S.E. (*A*) and increases (*B*) in lobar arterial pressure with i.a. depressor and pressor agents, respectively, injected before (*open bars*) and after (*solid bars*) PNU-37883A (5 mg/kg i.v.). The data demonstrate that, in this vascular bed, PNU-37883A blocks the vasodilator responses to levcromakalim, cromakalim, and pinacidil and potentiates the vasoconstrictor responses to U-46619, PGD_2, $PGF_{2\alpha}$, and Bay K 8644. $^*P \leq 0.05$ from control; $n = 4$ to 11 cats/group. From ref. 28, with permission.

scald injury, isoproterenol, and nitroglycerin was unaffected by PNU-37883A (112). Sabates et al. (97) reported that PNU-37883A (1 mg/kg i.c.a.) blocked the coronary dilator responses to pinacidil, adenosine and human adrenomedullin, but did not affect those induced by calcitonin gene-related peptide or nitroglycerin. PNU-37883A has also proven useful in conscious dogs, where a 6-h 6-μg/kg/min i.v. infusion prevented minoxidil (0.5 mg/kg) hypotension and seemed to block the K-ATP channel opener component of the antianginal agent nicorandil (54). Collectively these many investigations further contribute to PNU-37883A's acceptance as a relatively selective, easily formulated, long-acting, nonhypoglycemic vascular K-ATP channel blocker useful in preventing and/or reversing the vasodilator effects of structurally diverse K-ATP channel openers.

Renal Function

Vallon et al. (113) have used PNU-37883A to explore the urinary excretion and plasma renin activity effects of K-ATP modulators under various dietary K^+ conditions. In anesthetized rats on a normal 0.7% K^+ diet, PNU-37883A (15 mg/kg i.v.) again proved diuretic and natriuretic independent of K^+ excretion or glomerular filtration rate. The mean arterial pressure was stable, the heart rate declined, and like in dogs (52), the plasma renin activity declined 55%. Conversely, 10 μg/kg of the potent cyanoguanidine K-ATP channel opener P-1075 induced hypotension, tachycardia, electrolyte retention, and hyperreninemia. Relative to these basal conditions, a high K^+ diet (2%) attenuated PNU-37883A's natriuresis, while a low K^+ diet (0.04%) virtually eliminated it, possibly because of diet-induced changes in the renal tubular K-ATP channel density or open probability. Again like i.r.a. PNU-37883A in dogs (52), the drug also seemed to directly suppress juxtaglomerular renin release under non-natriuretic conditions.

Vallon et al. (114) also used PNU-37883A to explore the role of luminal K^+ in controlling renal tubuloglomerular feedback in rats. These investigators first confirmed that 100 μM PNU-37883A blocks Na^+ and fluid reabsorption and K^+ secretion in the microperfused cortical collecting duct (116). They then used this drug concentration to prevent K^+ entry into retroperfused tubules as a means of gauging the impact of tubular flow and K^+ on macula densa-mediated changes in the single nephron glomerular filtration rate. When combined with a K^+-free perfusate, PNU-37883A kept luminal K^+ levels extremely low and blocked the normal 14-nL/min reduction in adjacent single nephron glomerular filtration, thereby suggesting that K^+ signaling via the macula densa contributes to tubuloglomerular feedback. Verifying this result, single nephron glomerular filtration again fell as per the control circumstance when 5 μM KCl was added to the perfusate + PNU-37883A combination. It thus was concluded that intraluminal K^+ reaching the macula densa, which is controlled by the $Na^+/2Cl^-/K^+$ cotransporter and low-conductance K-ATP channels in the thick ascending limb of the loop of Henle, helps to control single nephron glomerular filtration, and that a minimal K^+ level is necessary in this nephron segment to trigger the tubuloglomerular feedback response.

PHARMACOKINETICS

Only limited pharmacokinetic data have been generated with PNU-37883A. A flame ionization gas chromatographic technique was developed to measure the free base in urine

and plasma (Wickramasinge and Shaw, unpublished data). Samples were taken to pH 12.7, extracted with methylene chloride, acidified to pH 1.8 with HCl in ether, re-evaporated, and reconstituted in 10 μL of chloroform. Specimens and an internal standard (analog PNU-37077) were then assayed with a Varian Acrograph gas chromatograph equipped for flame ionization. Unchanged PNU-37883 had a 6-min retention in a glass column packed with 0.5% carbowax (20 M) on 80-100 mesh glass beads with N_2 (100 mL/min). The lower level of detection was 20 ng/mL and was linear through 200 ng/mL.

With this assay, the plasma levels and urinary excretion of PNU-37883A were monitored in a conscious dog 3.5 h after a short i.v. infusion of 10 mg/kg. A peak plasma concentration of 3.1 μg/mL was detected at 20 min, it had a biphasic decay curve (initial and secondary half-lives of 19 and 77 min, respectively). Over the entire test only 2.38 mg (1.7%) of unchanged urinary PNU-37883 were collected, the majority of which was excreted during the first 30 min. Two possible urinary metabolites were isolated but never identified. The drug's low peak plasma concentrations and urinary excretion were of concern, but additional kinetic studies were not pursued once development was terminated.

TOXICOLOGY

Because PNU-37883A never advanced to human testing, no formal toxicology studies have been conducted. Early in its development, stepped i.v. doses of PNU-37883A proved lethal in a conscious uninstrumented dog and rhesus monkey at a total of 45 mg/kg, presumably because of the myocardial depression later identified in intact animals (55), isolated rat hearts (53), and rabbit myocardium (55). Despite the limitations imposed by its cardiodepression, PNU-37883A is safely tolerated in experimental animals at doses acutely antagonizing vascular K-ATP channel openers ($\leq$5 mg/kg) and inducing a relatively eukalemic diuresis (5 to 15 mg/kg).

SUMMARY

PNU-37883A is a newly identified blocker of vascular, renal tubular, and neuronal ATP-sensitive potassium channels with a rather complex pharmacological profile. In isolated blood vessels, PNU-37883A selectively blocks K-ATP channel opener-mediated vasorelaxation and $^{42}K^+$ efflux, seemingly through non-sulfonylurea binding sites having functional synergy with glyburide. Electrophysiological studies confirm that micromolar concentrations of PNU-37883A block ATP-sensitive K^+ channels in *Xenopus* oocyte follicular, dog coronary artery, and rat mesenteric artery cells, but unlike glyburide, the drug has negligible effects on pancreatic beta (RINm5F), cardiac, or skeletal muscle K-ATP channels. Fifty- to 100-fold higher PNU-37883A concentrations also block rat renal tubular K-ATP channels in the apical membranes of principal cells isolated from the thick ascending limb of the loop of Henle and in the cortical collecting duct, in a manner akin to glyburide. Similar PNU-37883A concentrations also block the hepatic mitochondrial K^+ uniport, increase hepatic endoplasmic reticulum phosphatidylserine synthesis and Ca^{2+} permeability, and depress myocardial contractility, conduction, and contractile threshold, although these latter cardiac effects do not seem to be directly related to

sarcolemmal K-ATP blockade. While devoid of significant behavioral effects, submicromolar PNU-37883A also blocks a dopamine D_2 receptor-activated K^+ current in rat caudate-putamen neurons more potently than does glyburide. Consistent with these functional and electrophysiological effects, low-dose PNU-37883A ($\leq$5 mg/kg i.v.) prevents and reverses the arterial vasodilation and hypotension of chemically diverse K-ATP channel openers *in vivo,* with little effect on plasma glucose and only minor cardiovascular changes. Slightly higher i.v. (5 to 15 mg/kg) and p.o. (10 to 30 mg/kg) doses of PNU-37883A exert a relatively eukalemic diuresis free of the K^+ imbalance and hyperreninemia characteristic of standard diuretics, reflecting the biological activity for which the drug was initially developed. Higher systemic doses of PNU-37883A markedly depress cardiac function. Thus, PNU-37883A and its closely related morpholinoguanidines represent new, non-sulfonylurea K-ATP channel blockers that can be used to block neuronal, vascular, and renal tubular K-ATP channels, thereby serving as valuable alternatives to glyburide and 5-hydroxydecanoate for exploring K-ATP control of these cellular functions.

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