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In Vitro and *in Vivo* Evaluation of an Endothelin Inhibitor Reveals Novel K^+ Channel Opening Activity

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A low molecular weight endothelin (ET-1) inhibitor (Ex. 127, European Patent Application 404 525 A2, Takeda Chemical Ind., 1991), CGS 26061, was synthesized and evaluated to determine its mechanism of action. CGS 26061 (10 μM) failed to inhibit binding of [^{125}I]ET-1 in porcine thoracic aorta and was without effect on ET-1-induced [^{3}H]inositol phosphate accumulation in A7r5 cells. However, CGS 26061 relaxed porcine coronary arterial rings precontracted with ET-1. In addition, contractions to $PGF_{2\alpha}$ and low K^+ (20 mM) but not high K^+ were attenuated, suggesting that CGS 26061 (1, 10 μM) is a potassium channel opener. Patch-clamp experiments confirmed the K^+ channel activity (0.1-10 μM). The originally re ported inhibition of ET-1-induced pressor responses by Ex. 127 (CGS 26061) was not replicated in the anesthetized dog or conscious rat nor was it shown to be antihypertensive in SHR. These data have identified CGS 26061 as a novel K^+ channel opener with a unique cardiovascular profile.

Endothelin, a 21 amino acid peptide, is one of the most potent vasoconstrictor peptides known (1). Initial studies using ET-1 and structurally similar peptides of the ET family (2), have implicated the ET's in a variety of pathophysiological states, including hypertension (3,4) and renal disease (5,6). Recently, the use of specific endothelin receptor antagonists have further substantiated a role for endothelin in acute (7,8) and chronic renal failure (9) as well as in cerebral vasospasm (10). The cellular mechanisms responsible for the actions of ET-1, although not precisely defined, have been extensively evaluated and primarily result from a rise in intracellular calcium ($[Ca^{2+}]_i$) (1,11,12). Therefore, it is expected that agents which prevent an increase in $[Ca^{2+}]_i$, will to some extent, functionally inhibit the actions of ET-1. Recently, an organic low molecular weight compound, 9-phenyl-7-propyl-4H,6H-pyrimido[6,1-b][1,3]-thiazine-6,8(7H)-dione (Ex. 127), was identified as a potent ET-1 ''inhibitor'' (Takeda Chemical Ind., 13). The objective of the present study was to define the specific mechanism by which Ex. 127 inhibits the actions of ET-1. This compound was therefore synthesized in-house for experimental evaluation (Ex. 127 synthesized by Chemistry Research, Ciba Pharmaceuticals, Summit, NJ and designated CGS 26061).

MATERIALS AND METHODS

In Vitro Pharmacology

Receptor binding. Porcine thoracic aorta and rat lung were rinsed twice with modified Krebs-Ringers solution, cut into 4 mm squares, homogenized using a Polytron and centrifuged at 1000 × g for 15 min. The supernatant was filtered through a double-layered nylon mesh and centrifuged at 48,000 × g for 30 min. Pellets were resuspended in 10 vols Krebs buffer and centrifuged three times. The final pellets were stored at −70°C. Endothelin ET_A and ET_B receptor binding experiments were performed with CGS 26061 as previously described (14).

PI hydrolysis measurements. The release of [^{3}H]inositol phosphates from A7r5 cells was measured as previously described (15).

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Electrophysiology. Single smooth muscle cells from porcine coronary arteries were prepared by incubation of the arterial media-intimal layer in Ca^{2+} free saline containing 0.2% (w/v) collagenase, 0.2% papain and 0.04% dithiothreitol for 55 min at 37°C. Single cells were suspended in a ''KB'' solution (16) for 1 hr before experiments. K^+ currents through single channel (cell-attached) and entire cell membrane (whole-cell) were recorded using the tight-seal patch-clamp method (17). In single channel recordings, the bath and pipette solutions were identical and were composed of (mM) KCl, 140; $MgCl_2$, 1; $CaCl_2$, 1; NaH_2PO_4, 5; glucose, 5; HEPES, 10; pH 7.4. In whole-cell recordings, the bath solution contained (mM) NaCl, 135; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 5 and HEPES 10; pH 7.3. The pipette solution had a composition of (mM) KCl, 140; $MgCl_2$, 1; Na_2UDP, 2; K_2ATP, 2; $CaCl_2$, 0.1; EGTA, 0.6; glucose, 5; HEPES, 10, pH 7.3. Recording arrangements and patch-clamp electronics were described elsewhere (18).

Isometric tension measurements. Arterial rings isolated from left anterior descending coronary artery of porcine heart were suspended in water-jacketed tissue baths (20 ml) filled with normal physiological saline (NPS) at 37°C and continuously aerated with 5% CO_2 in O_2. NPS was of the following composition (in mM): NaCl, 120; KCl, 5; KH_2PO_4, 1; $MgSO_4$, 1.6; $CaCl_2$, 1.6; $NaHCO_3$, 25; and dextrose, 10.8. A resting tension of 4 g was applied to the tissue and the muscle was equilibrated for a minimum of 90 min in NPS before experimental procedures were begun. In some experiments, the endothelium was mechanically disrupted by abrasion of the lumen of the vessel with a roughened wooden applicator.

Each arterial ring was contracted twice with added KCl (20 mM). Integrity of the endothelium was functionally tested by assessing the effects of substance P (0.1 mM) on tone induced by 3 mM $PGF_{2\alpha}$. The arterial rings were then contracted with a single dose (EC_{80}) of ET-1 (3 nM), $PGF_{2\alpha}$ (3 mM), or K^+ (20 mM). When isometric tension reached a steady state, tissues were exposed to CGS 26061. Subsequent doses were cumulatively added only after tension had reached a steady state. Control tissues received equivalent amounts of vehicle (DMSO). Percent relaxation induced by CGS 26061 was calculated relative to the plateau of contractile force that was achieved with the respective contractile substance.

In Vivo Pharmacology

Conscious rat. Male Sprague-Dawley rats (Tac:N(SD)fBR) or spontaneously hypertensive rats (Tac:N(SHR)fBR) weighing 340-390 g were used in these experiments (Taconic Farms, Germantown, NY). Two days prior to experimentation, under methoxyflurane anesthesia, rats were instrumented with femoral arterial and venous catheters for measurement of mean arterial blood pressure (MAP) and heart rate (HR) and for injection of drugs, respectively. Cardiovascular responses to ET-1 (0.5 nmol/kg i.v.) in the absence of CGS 26061 (control) were compared to those in the presence of CGS 26061 (1 mg/kg i.v.) or vehicle (42.5% ethanol). SHR were implanted at least 2 weeks prior to experimentation with radiotelemetric implants as previously described (19). CGS 26061 (10, 30 mg/kg) or EMD 52692 (Ref. 20; 30, 100 μg/kg) were administered to conscious SHR by oral gavage. Mean arterial pressure and HR were monitored continuously in rats for a period of up to 8 hours after oral dosing.

Anesthetized dog. Experiments were conducted in six mongrel dogs anesthetized with pentobarbital sodium (30 mg/kg i.v.). Animals were ventilated with room air and arterial blood gases and pH were maintained within normal limits. Rectal temperature was monitored and maintained within $\pm$ 0.5°C of baseline temperature. Cannulae were inserted into a femoral artery and vein for measuring arterial pressure and for administering compounds, respectively. After hemodynamic stabilization, ET-1 (0.1 nmol/kg i.v. bolus) was injected to determine the control arterial pressure response. Thirty min later, either CGS 26061 (1 mg/kg i.v.) or its vehicle (0.4 ml/kg i.v.; 50% ethanol in saline) was administered, followed 5 min later by a second injection of ET-1.

Statistical and data analysis. All data were expressed as means $\pm$ SE. Statistical significance was determined by application of an analysis of variance, followed by Newman-Keuls multiple range test for comparison of means. Differences with $p<0.05$ were considered significant. Student's t-test were used to determine differences between control and treatment groups.

RESULTS AND DISCUSSION

In the porcine thoracic aorta, CGS 26061, at a concentration ranging from 0.001-100 μM, inhibited less than 15% specific [^{125}I]ET-1 binding. In contrast, the IC_{50} value for ET-1 was 0.17 $\pm$ 0.005 nM and 47 $\pm$ 3 nM for ET-3 (n=3) indicating that [^{125}I]ET-1 labels the ET_A receptor in porcine aorta (21). In rat lung, CGS 26061 was unable to inhibit more than 35% specific binding of [^{125}I]ET-3. The IC_{50} values for ET-1 and ET-3 were 0.074 $\pm$ 0.003 and 0.24 $\pm$ 0.02 nM, respectively (n=3), indicating that [^{125}I]ET-3 binds to the ET_B receptor in rat lung (22). Thus, our results indicate that the ''ET-1 inhibitory'' effects of this agent are not mediated by receptor blockade but more likely reflect a functional antagonism.

One such mechanism whereby a functional antagonism of ET-1-induced effects could occur is through interruption of the inositol phosphate cascade. ETs have been reported to stimulate

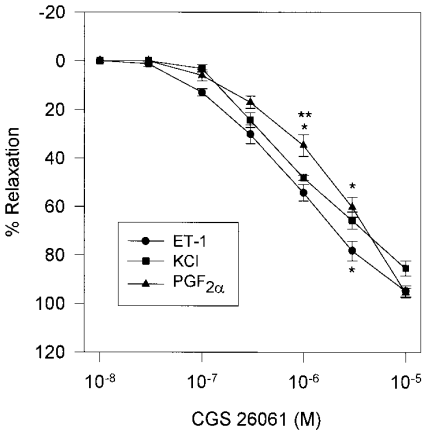

FIG. 1. Effect of CGS 26061 on contractions induced by 3×10^{-9} ET-1 (n=6), 20×10^{-3}M added K^+ (n=4), and 3×10^{-6}M $PGF_{2\alpha}$ (n=5) in isolated porcine coronary arterial rings. Relaxation produced is expressed as a percentage of the contractile force induced by the agonist. Each point represents the mean ± S.E. *Significantly different from ET-1 response; **significantly different from K^+ response; $p<0.05$.

phospholipase C-mediated phosphoinositide (PI) hydrolysis in a variety of tissues and cultured cells, resulting in the generation of the second messengers, inositol trisphosphate and diacylglycerol (23,24,25). However, CGS 26061 (0.01-10 μM) did not affect [^{3}H]inositol phosphate accumulation induced by 5 nM ET-1 nor did it stimulate PI hydrolysis when added alone to intact A7r5 cells.

CGS 26061 was further shown to cause concentration-dependent inhibition of ET-1-, $PGF_{2\alpha}$- and K^+-induced contractions in endothelial intact or denuded porcine coronary arteries (Fig. 1). The maximum relaxation induced by 10 μM CGS 26061 was, however, similar with each of the 3 contractile agents tested; 94.8 ± 2.5% with ET-1 (n=6), 85.4 ± 3.6% with K^+ (n=4), and 95.5 ± 2.2% (n=5) with $PGF_{2\alpha}$. These data are in agreement with previous results (13) and provide additional information demonstrating a lack of specificity of this compound to inhibit ET-1-induced contractions. In addition, CGS 26061 effectively relaxed vessels precon tracted with low K^+ (20 mM) but not high K^+ (80 mM), indicative of a potassium channel opener (26).

We examined the direct effects of the compound on the whole-cell K^+ current and the activity of single K^+ channels in smooth muscle cells isolated from porcine coronary arteries. A typical response to CGS 26061 of the whole-cell K^+ current elicited by depolarization steps is shown in the left panel of Fig. 2. The compound induced a concentration-dependent augmentation of the amplitude of the K^+ current. The phenomenon is illustrated in the current-voltage relationship in the right panel of Fig. 2. Judging from the current traces and the current-voltage relationship, it was evident that the stimulatory effect occurred mainly at higher depolarizing voltages where the large-conductance Ca^{2+}-activated K^+ channels were active. Fig. 3 shows an activation by CGS 26061 of the single Ca^{2+}-activated K^+ channel in a cell-attached patch. The channel open-state probability calculated from prolonged recordings was 0.6% in control and 2.0% in 1 μM CGS 26061, which resulted from a 31% increase in channel mean open-time and a concomitant 60% decrease in the mean closed-time.

In conscious normotensive rats, injection of ET-1 (0.5 nmol/kg i.v.) initially produced a transient decrease in blood pressure followed by a more prolonged increase, reaching a maximum above baseline of 37 ± 3% (baseline mean arterial pressure = 116 ± 2 mmHg) 10 min after administration of ET-1. A decrease in heart rate of 29 ± 2% (baseline heart rate = 382

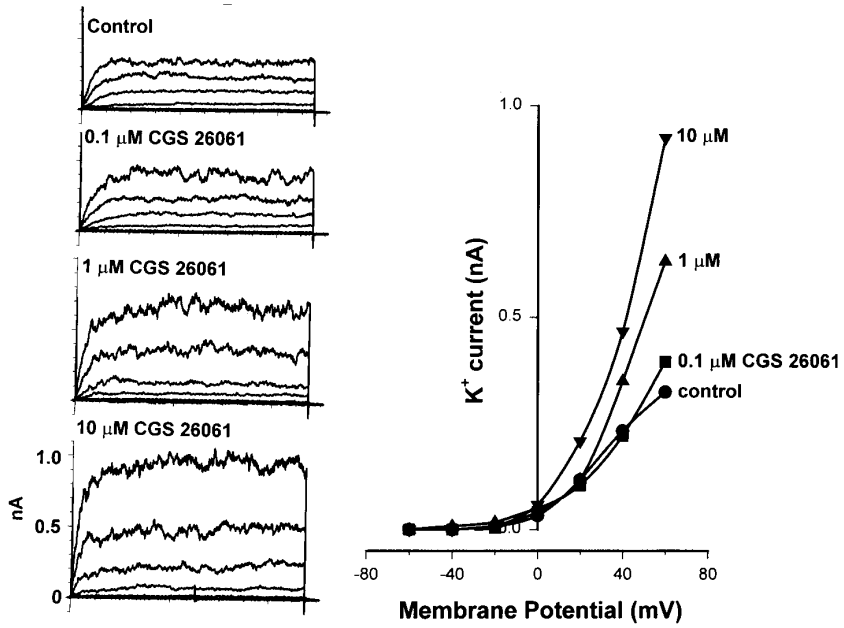

FIG. 2. Effect of CGS 26061 on the whole-cell K^+ currents. Left panel from top to bottom: A family of whole-cell K^+ current in control, in 0.1, 1 and 10 mM CGS 26061. The currents were elicited by depolarization steps ranging between −60 and +60 mV with an increment of 20 mV. Holding potential was −60 mV and the pulse duration was 1 sec. Right panel: current voltage curves in control (circle), in the presence of 0.1 μM (square), 1 μM (triangle) and 10 μM CGS 26061 (inverse triangle).

± 2 beats/min) accompanied the pressor response. The ET-1-induced pressor response in animals receiving CGS 26061 (1 mg/kg), although slightly attenuated compared to control, was not different from that observed in vehicle-treated (42.5% ethanol) rats. Baseline mean arterial pressure in normotensive rats was not affected by CGS 26061 administration, 5 minutes prior to ET-1 administration. Furthermore, oral administration of CGS 26061 to conscious-telemetered SHR at doses up to 30 mg/kg, failed to alter mean arterial pressure (Fig. 4), whereas another K^+ channel opener, EMD 52692, elicited a pronounced antihypertensive effect.

In six dogs, ET-1 caused a transient decrease in arterial blood pressure followed by a slowly developing and more sustained pressor response. Before the administration of CGS 26061, arterial pressure decreased by 42 ± 9 mmHg from a baseline of 113 ± 5 mmHg, then increased by 13 ± 5 mmHg above baseline (n=4). Five minutes after CGS 26061 administration, baseline arterial pressure was not significantly altered. However, the depressor response to ET-1 was blunted by 56% whereas the pressor response was unchanged. Vehicle administration in two additional dogs did not alter baseline arterial pressure or the depressor and pressor responses to ET-1.

Our results demonstrate a vasorelaxant effect of CGS 26061 in ET-1 contracted porcine coronary arteries and are in agreement with previous findings (13). The present experiments further demonstrate that the vasorelaxant actions of CGS 26061 are likely due to K^+ channel opening activity, and not due to inhibition of ET at the receptor and/or second messenger level. The pattern and magnitude of cardiovascular responses evoked by ET-1 in conscious rats and in anesthetized dogs in the present study were similar to those previously reported (13,27,28). However, our results with this purported ''endothelin inhibitor'' are in conflict

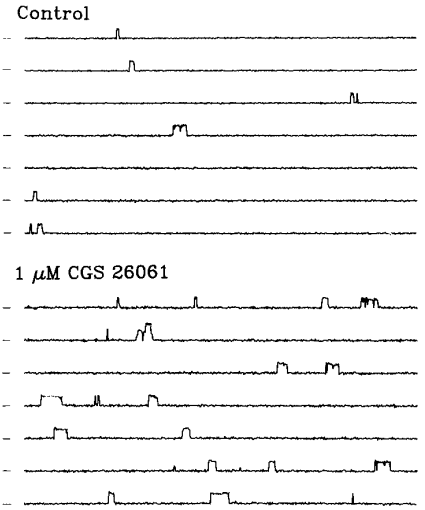

FIG. 3. Effect of CGS 26061 on the activity of the Ca^{2+}-activated K^+ channel in a cell-attached patch. Single channel recordings in control (upper) and in the presence of 1 μM CGS 26061 (lower). [The channel has a conductance of 214 $\pm$ 6 PS (n=16) in symmetrical K^+ solutions.] Seven traces in each panel are consecutive recordings, each with a duration of 1 sec. Upward deflections are outward channel opening events. Short lines on the left side of traces indicate channel closed state. Data were filtered at 2 kHz.

with those elicited in the conscious beagle dog in which the authors reported an 86% and 100% attenuation of the depressor and pressor responses to ET-1, respectively (13). The reasons for these differences remain unresolved. The inability of CGS 26061 to block the pressor effects of ET-1 *in vivo* is not likely due to species differences or anesthetic, since in the present study, both the anesthe tized dog and conscious rat were evaluated. We conclude that CGS

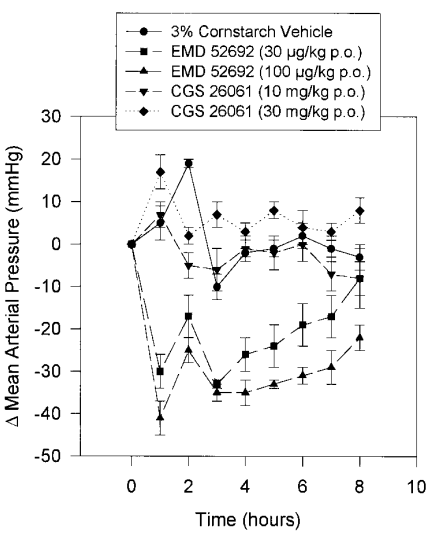

FIG. 4. Conscious-telemetered SHR received a single oral dose of either CGS 26061 (10, 30 mg/kg) or EMD 52692 (30, 100 μg/kg) and mean arterial pressure was monitored continuously for 8 hr. Arterial pressure significantly decreased following EMD 52692 (2-way ANOVA for repeated measures, *post-hoc* test, Student-Newman-Keuls; $p < 0.05$) while CGS 26061 was ineffective.

26061 possesses a unique K^+ channel opener profile. It was a potent vasorelaxant of coronary vessels while having no significant peripheral vascular effects as indicated by the absence of a hypotensive or antihypertensive response in conscious and anesthetized animals.

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