Pharmacology of SB-273779, a Nonpeptide Calcitonin Gene-Related Peptide 1 Receptor Antagonist

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ABSTRACT
Calcitonin gene-related peptide (CGRP), a potent vasodilatory and cardiotoxic peptide, has a potential role for CGRP in diverse physiologic and pathophysiologic situations such as congestive heart failure, diabetes, migraine, and neurogenic inflammation. Although a peptide CGRP receptor antagonist, CGRP$_{\text{A}}$ is available, its utility presents significant limitations for these indications. Here, we describe the properties of SB-(++)-273779 [N-methyl-N-[2-methylphenyl]-3-nitro-4-[2-thiazolylsulfinyl]nitosobenzanilide], a selective nonpeptide antagonist of CGRP$_1$ receptor. SB-(++)-273779 inhibited $^{125}$I-labeled CGRP binding to SK-N-MC (human neuroblastoma cells) and human cloned CGRP$_1$ receptor with $K_i$ values of 310 ± 40 and 250 ± 15 nM, respectively. SB-(++)-273779 also inhibited CGRP (3 nM)-activated adenylyl cyclase in these systems with IC$_{50}$ values of 390 ± 10 nM (in SK-N-MC) and 210 ± 16 nM (recombinant human CGRP receptors). Prolonged treatment (>30 min) of SK-N-MC cells with SB-(++)-273779 followed by extensive washing resulted in reduction in maximum CGRP-mediated adenylyl cyclase activity, suggesting that this compound has irreversible binding characteristics. In addition, SB-(++)-273779 antagonized CGRP-mediated 1) stimulation of intracellular Ca$^{2+}$ in recombinant CGRP receptors in HEK-293 cells, 2) inhibition of insulin-stimulated [14C]deoxyglucose uptake in L6 cells, 3) vasodilation in rat pulmonary artery, and 4) decrease in blood pressure in anesthetized rats. SB-(++)-273779 tested at 3 µM had no significant affinity for calcitonin, endothelin, angiotensin II, and α-adrenergic receptors under standard ligand binding assays. SB-(++)-273779 also did not inhibit forskolin and pituitary adenylate cyclase-activating polypeptide. These results suggest that SB-(++)-273779 is a valuable tool for studying CGRP-mediated functional responses in complex biological systems.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide that is synthesized from alternate processing of the calcitonin gene mRNA (Amara et al., 1982). CGRP is produced in sensory neurons and is transported peripherally to terminal areas in visceral organs and centrally to the dorsal horn. In the cardiovascular system, CGRP is a potent vasodilator in several species (Bell and McDermott, 1996) and produces an increase in heart rate (Franco-Cereceda, 1988). CGRP has a positive inotropic effect on isolated rat ventricular cardiomyocytes (Bell and McDermott, 1994) and in isolated trabeculae from porcine right atria and left ventricles (Saetrum Opgaard et al., 1999). CGRP mediates its action via two functional organ receptors that are primarily coupled to the activation of adenylyl cyclase. CGRP receptors have been identified and characterized from several tissues of neuronal and peripheral origin. CGRP mediates its action via two functional receptor subtypes, the CGRP$_1$ and the CGRP$_2$ receptor (Dennis et al., 1989). The fragment CGRP$_{8-37}$ is a selective functional antagonist for CGRP$_1$ receptors. Reduction of the disulfide bond of CGRP, which destroys the N-terminal ring structure of the peptide, yields a linear analog, diacetoamidomethyl cysteine CGRP ([Cys (ACM)$_2$,7] CGRP), that is a selective agonist for CGRP$_2$ receptors. CGRP$_1$ receptors have been cloned from human (Aiyar et al., 1996), rat (Han et al., 1997), and pig (Elshourbagy et al., 1998). They show 91 to 95% identity at the amino acid level among species. The receptor is a seven-transmembrane domain receptor protein, which belongs to a subgroup of the G-protein-coupled receptor family, and is predominantly expressed in heart and lung.
(Dang et al., 1999). The pharmacological profile of the cloned CGRP receptor is quite similar to the endogenous CGRP₁ receptor present in human neuroblastoma cells, SK-N-MC (Aiyar et al., 1996). These receptors are coupled to production of cAMP, which is regarded as the major mechanism for smooth muscle relaxation and vasodilation caused by CGRP. In addition, CGRP can also activate phospholipase C and thereby stimulate the intracellular calcium concentration ([Ca²⁺]). (Aiyar et al., 1999). Interest has also been generated about the role of CGRP in insulin-mediated glucose metabolism. CGRP has been shown to inhibit both insulin secretion and actions in vitro (Wimalawansa, 1997). In skeletal muscle, CGRP impairs glycogen synthesis and enhances glycogenolysis, glycolysis, and lactate production. CGRP, like angiotensin, decreases basal and insulin-stimulated glycogen synthesis in muscle and stimulates lactate production from isolated soleus muscle. Taken as a whole, these results indicate a potential role for CGRP to the pathophysiology of type 2 diabetes.

Evidence is accumulating that inappropriate release of CGRP is a potential causative factor in several diseases, including migraine, type 2 diabetes, inflammation, and congestive heart failure. These postulations are derived from the findings that increased concentrations of immunoreactive CGRP are present in the circulation (Wimalawansa, 1997). To determine the endogenous role of CGRP, investigators have used the CGRP receptor peptide antagonist, CGRP₈₋₃₇, with limited success because of its peptidic nature. CGRP₈₋₃₇ have used the CGRP receptor peptide antagonist, CGRP₈₋₃₇, to determine the endogenous role of CGRP, investigators have used the CGRP receptor peptide antagonist, CGRP₈₋₃₇, with limited success because of its peptidic nature. CGRP₈₋₃₇ displays a wide range of affinities against CGRP responses across different species and tissues. The apparent pA₂ values of CGRP₈₋₃₇ in various assays are highly variable, with values ranging between 6.0 to greater than 9.0. It is also known that CGRP₈₋₃₇ had vasodilator activity at micromolar concentration in the pulmonary artery, indicating partial agonistic property (Wisskirchen et al., 1998). Identification of a nonpeptide CGRP antagonist would provide an excellent tool to study the role of CGRP. Such compounds will be a promising lead for novel therapeutic agents for the treatment of type-2 diabetes, migraine, and pain. Based on these considerations, we screened the SmithKline Beecham (SB) chemical collection bank for novel CGRP receptor ligands, and identified a class of benzamidines as potential selective CGRP receptor antagonists. In the present study, we have evaluated the pharmacology of SB+(+)273779 [N-methyl-N-(2-methylphenyl)-3-nitro-4-(2-thiazolylsulfanyl)nitrobenzanilide] (see Table 1) using endogenous and recombinant CGRP receptors. Our results confirm that SB+(+)273779 is a selective nonpeptide antagonist with submicromolar activity, which could prove useful in understanding the biology and pharmacology of CGRP₁ receptor.

### Experimental Procedures

**Materials.** Human αCGRP (hαCGRP), human αCGRP₈₋₃₇ (hαCGRP₈₋₃₇), and endothelin were purchased from Bachem Bioscience (King of Prussia, PA). [2-¹²⁵I]Iodoacetidyl[¹⁴]hαCGRP (specific activity 2000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Chicago, IL). SB+(+)273779 and its related compounds were synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Generation of recombinant human and porcine CGRP receptors in HEK-293 cells has been previously described (Aiyar et al., 1996; Elshourbagy et al., 1998).

**Cell Culture.** SK-N-MC human neuroblastoma cells were obtained from the American Type Culture Collection and grown in T150 flasks at 37°C in the presence of 95% O₂/5% CO₂. HEK-293 cells, stably transfected with the human or porcine CGRP receptor cDNA, were maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum. At confluence, the cells were harvested by scraping followed by centrifugation at 2000g for 10 min at 4°C. The cell pellets were frozen at –70°C for receptor binding and adenylyl cyclase assays.

**Membrane Preparation.** The frozen pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM Na-EDTA and homogenized using a Dounce glass homogenizer. The homogenate was centrifuged for 20 min at 12,000g at 4°C, and the resultant membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂ and recentrifuged. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂ and assayed immediately.

**Radioligand Binding.** [¹²⁵I]CGRP binding to SK-N-MC cell membranes was performed using 20 nM Tris-HCl buffer at pH 7.4 containing 5 mM MgCl₂, 0.2% BSA and 0.1 mg/ml bacitracin as previously described (Semark et al., 1992). The assay was done in 500 μl of buffer containing 50 pM [¹²⁵I]CGRP, test compounds in the concentration range of 10 nM to 30 μM and 30 to 40 μM of membrane proteins. Various concentrations of test compound were made in dimethyl sulfoxide (DMSO) at 50-fold excess. Saturation binding experiments were carried out with increasing concentrations of [¹²⁵I]CGRP (5–120 pM) and 40 to 60 μg of membrane protein in the absence or presence of SB-273779 (100 and 300 nM). After the binding assay was done for 30 min at 25°C, the reaction mixture was rapidly diluted with 2 ml of cold wash buffer (0.9% NaCl) followed by rapid filtration over Skatron Filter Mates presoaked in 0.2% polyethyleneimine using a Skatron cell harvester (Skatron Instruments, Lier, Norway). All binding assays were done in duplicate, and each experiment was repeated three to four times. Analysis of all binding data (i.e., the determination of Kᵦ, Bmax and Kᵥ values) were performed using computer-assisted nonlinear least square fitting using PRIZM (GraphPad Software, Inc., San Diego, CA). Nonspecific binding was defined using hCGRP at a final concentration of 1 μM. The radioactivity was determined using a gamma counter (Packard Instrument, Meriden, CT).

**Adenylyl Cyclase Assay.** Membrane-bound adenylyl cyclase activity was determined as the rate of conversion of [α-³²P]ATP to [³²P]cAMP as previously described (Elshourbagy et al., 1998). Membranes (40–60 μg of protein), various concentrations of test compounds (10 nM to 10 μM), and 5 nM CGRP were incubated in triplicate tubes in buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1.2 mM ATP, 0.1 mM cAMP, 2.8 mM phosphoenolpyruvate, and 5.2 μg/ml myokinase in a final volume of 100 μl for 20 min at 30°C. Experiments were also done to measure the adenylyl cyclase activity in the absence of CGRP (basal activity) and test compound by itself at 1 and 10 μM. The reactions were stopped with 1 ml of a solution containing 0.28 mM cAMP, 0.33 mM ATP, and 22,000 dpm [³²P]cAMP. [³²P]cAMP was separated using sequential chromatography on Dowex and alumina columns (Salmon et al., 1974). The results are expressed as percentage of inhibition of CGRP-mediated adenylyl cyclase activity at various concentrations of test compounds.

**Intracellular [Ca²⁺], Mobilization.** CGRP-meditated intracellular [Ca²⁺], mobilization was studied using recombinant porcine CGRP receptor stably expressed in HEK-293 cells as discussed (Aiyar et al., 1999). The effect of SB-273779 (at 0.1, 1.0, and 10 μM) was studied on basal and CGRP-mediated [Ca²⁺], mobilization in recombinant porcine CGRP receptor.

**[¹⁴]C]Deoxyglucose Uptake.** L6 cells were obtained from the American Type Culture Collection and grown in 6-well plates in MEM containing 2% bovine serum. Experiments were done following the procedure of Kreutter et al. (1989). In brief, cells were serum-
depleted for 5 h in αMEM. During the final 60 min of this period, the cells were treated with insulin (1 μM) or insulin (1 μM) plus SB-273779 (10 μM) or CGRP (10 nM) or insulin (1 μM) plus CGRP (10 nM) and SB-273779 (1 μM). The cells were washed, and uptake of 0.1 mM [1-14C]2-deoxyglucose was initiated. Incubations were performed in triplicate for 10 min at room temperature. Uptake was terminated by aspirating the buffer and washing the cells with ice-cold PBS. Cells were dissolved in 0.1 N NaOH and counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

**Vasorelaxation Measurements in Pulmonary Artery.** All animals were housed in an accredited laboratory animal facility, and all procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health, Education, and Welfare; Department of Health and Human Services publication NIH 85-23). The Animal Care and Use Committee at SmithKline Beecham Pharmaceuticals approved all procedures. Male Wistar rats (275–300 g, Janvier, Le Genest, France) were anesthetized with sodium thiopentone (50 mg/kg i.p., Nesdonal, Rhône Mérieux, Lyon, France) before excision of the pulmonary artery. The vessel placed in cold Krebs-Henseleit solution oxygenated with 95% O2/5% CO2 mixture at 37°C. The composition of the Krebs-Henseleit solution was as follows (in mM): NaCl (95), KCl (5), CaCl2 (2.6), MgSO4 (1.2), KH2PO4 (1.2), NaHCO3 (24.9), and D-glucose (10), pH 7.4. The tension was measured isometrically using a force transducer (EMKA Technologies, Paris, France) and recorded on chart paper (RS-3400, Gould Instruments, Ballainvilliers, France). After equilibration for a minimum period of 45 min, at an optimal resting tension of 0.7 g, each ring was precontracted with repeated application of phenylephrine (0.1 μM). The functional integrity of the endothelium was evaluated by assessing the relaxation induced by 1.0 μM acetylcholine in preconstricted vessels. Test drugs were then applied at the maximum contraction induced by phenylephrine, and the vasorelaxing effect of CGRP (3.0 nM) was evaluated as the percentage decrease of the maximal contraction caused by phenylephrine. The inhibitors were applied 20 min before application of CGRP. The reversibility of inhibitory action of compounds on the vasorelaxation was evaluated after washing the preparations and challenging them with CGRP at 20-min intervals.

**Acute Blood Pressure Monitoring in the Anesthetized Rat.** Male Sprague-Dawley rats weighing 350 to 380 g were used in experiments in vivo. The procedures employed were similar to those described previously (Willette and Sauermelch, 1990). Briefly, surgical anesthesia was induced with 3.0% isoflurane delivered in 100% O2. Polyethylene catheters were inserted in the left femoral artery and vein for the continuous monitoring of arterial blood pressure and the administration of drugs, respectively. Inhalation anesthesia was discontinued, and anesthesia was maintained by the intravenous administration of pentobarbital (40 mg/kg i.v.). Propranolol (1 mg/kg i.v.) and atropine sulfate (0.2 mg/kg i.v.) were administered to reduce reflex regulation of the heart. Supplemental doses of pentobarbital (10 mg/kg i.v.) were administered as needed. All animals breathed spontaneously, and no signs of respiratory distress were apparent. The duration of each experiment was ≤1 h. These preparations were used to determine the effects of treatment with SB-273779 (10 mg/kg i.v.) or vehicle (50% DMSO in 10% 2-hydroxypropyl-β-cyclodextran) on the hypotensive effect elicited by the bolus administration of CGRP (100 or 300 pmol/kg i.v.). CGRP was administered 10 min following completion of SB-273779 administration.

**Results**

High-throughput screening of SB-compound libraries led to the identification of a low-affinity lead, SB-211973 (3 μM), that served as a starting point for subsequent chemical modifications. Optimization of this activity by structural modification of the lead compound resulted in a series of nitrobenzaniides as represented by a racemic compound, SB-268262 (thiazolylsulfoxide nitrobenzanilide) (Table 1). The individual enantiomers of SB-268262 were separated via chiral HPLC (SB-(+)-273779 and SB-(-)-273780) and evaluated. SB-(+)-273779 was 5-fold more active than SB-(−)-273780 in the cyclase assay and 2-fold more active than

<table>
<thead>
<tr>
<th>SB-Number</th>
<th>Compound</th>
<th>Binding IC50</th>
<th>Cyclase IC50</th>
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<tr>
<td>SB-211973</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>1.93 ± 0.47</td>
<td>0.9 ± 0.37</td>
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<tr>
<td>SB-(±)-268262</td>
<td><img src="image2" alt="Chemical Structure" /></td>
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<td>0.83 ± 0.29</td>
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<tr>
<td>SB-(+)-273779</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>0.31 ± 0.04</td>
<td>0.39 ± 0.01</td>
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<tr>
<td>SB-(−)-273780</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>0.69 ± 0.19</td>
<td>2.07 ± 0.17</td>
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SB-(±)-268262. SB-(+)-273779 was selected to study its effect on the CGRP receptor. SB-(+)-273779 inhibited the specific binding of \([^{125}\text{I}]\text{CGRP}\) to CGRP1 receptors, with an inhibitory potency \((K_i)\) of 310 ± 40 and 250 ± 15 nM on membranes from the human neuroblastoma cell line SK-N-MC and recombinant human CGRP1 receptor in HEK-293 cells, respectively (Fig. 1). However, SB-(+)-273779 and its related compounds were weak in displacing \([^{125}\text{I}]\text{CGRP}\) binding from membranes isolated from rat or porcine lung and also recombinant porcine CGRP receptor expressed in HEK 293 cells, which are known to possess high-affinity CGRP binding site. We investigated the saturation binding of \([^{125}\text{I}]\text{CGRP}\) to the membranes from SK-N-MC in the absence or presence of SB-(+)-273779 (Fig. 2). Analysis of Scatchard transformation of the \([^{125}\text{I}]\text{CGRP}\) saturation curve in the presence of SB-(+)-273779 (0.3 and 1 μM) revealed that SB-(+)-273779 did not affect the total number of binding sites labeled by \([^{125}\text{I}]\text{CGRP}\) but increased the dissociation constant of the radioligand.

To determine whether SB-(+)-273779 is in fact a functional antagonist, its inhibitory effect on CGRP-activated adenyl cyclase, an event subsequent to receptor activation, was studied in SK-N-MC cells. These cell membranes responded to increasing concentrations of CGRP with EC50 values of 1 nM. CGRP (3 nM)-mediated adenyl cyclase activity was inhibited by CGRP8-37 with IC50 values of 60 nM. Similarly, SB-(+)-273779 also inhibited CGRP-mediated adenyl cyclase with IC50 values of 390 ± 10 nM (Fig. 3). SB-(+)-273779 did not alter the basal adenyl cyclase activity up to 1 μM, suggesting that there is an absence of agonistic activity for this compound. The compound displayed competitive antagonism against CGRP-mediated adenyl cyclase activity at lower concentration, whereas at 3 and 10 μM the maximum stimulation was decreased by 30% and 60%, respectively (Fig. 4). CGRP-activated adenyl cyclase activity was also determined for SK-N-MC cells pretreated with SB-(+)-273779 (3 μM) for different time points. The compound shifted the CGRP dose-response curve to the right suggesting competitive antagonism. However, prolonged treatment (>30 min) of the cells with the compound followed by extensive washing resulted in a significant decrease in CGRP-mediated maximum adenyl cyclase activity, suggesting this compound has irreversible binding characteristics (Fig. 5). The effect was observed for CGRP only because the compound did not affect vasoactive intestinal peptide (1 μM),

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**Fig. 1.** Displacement of \([^{125}\text{I}]\text{CGRP}\) binding by SB-(+)-SB-273779 on specific binding of \([^{125}\text{I}]\text{CGRP}\) to membranes prepared from SK-N-MC, recombinant human, and porcine CGRP receptor expressed in HEK-293 cells and porcine lung. Data are the means of duplicate measurements and are representative of three to five separate experiments.

**Fig. 2.** Scatchard analysis \([^{125}\text{I}]\text{CGRP}\) binding (20–250 pM) to SK-N-MC cell membranes in the absence and presence of SB-(+)-SB-273779. Data are the means of duplicate measurements and are representative of three separate experiments. Dissociation constant values were 22 pM (control) (■), 37 pM (in the presence of 0.3 μM SB-(+)-273779) (▲), 89 pM (in the presence of 1.0 μM SB-(+)-273779) (▼), and \(B_{\text{max}}\) was 220 fmol/mg of protein.

**Fig. 3.** Inhibition of CGRP (3 nM)-activated adenyl cyclase by SB-(+)-273779 in membranes prepared from SK-N-MC, recombinant human, and porcine CGRP receptors expressed in HEK-293 cells (HEK-293 PR and HEK-293 HR) and porcine lung. Data shown are representative of at least three separate experiments done in duplicate. PACAP, pituitary adenylate cyclase-activating polypeptide.

**Fig. 4.** Effect of SB-(+)-273779 (0.1, 1.0, 3.0, and 10 μM) on concentration-response curves of CGRP-activated adenyl cyclase in SK-N-MC cell membranes. Data shown are representative of three separate experiments done in duplicate.
isoproterenol (30 μM), and forskolin (10 μM)-mediated adenylyl cyclase activation (Fig. 5). SB-(+)-273779 exhibited similar inhibitory effect on CGRP-mediated activation of adenylyl cyclase in recombinant human CGRP receptors expressed in HEK-293 cell membranes. Despite the lack of inhibitory effect on [125I]CGRP binding in nonhuman CGRP binding studies (Fig. 1), SB-(+)-273779 effectively inhibited CGRP-mediated adenylyl cyclase activity in membranes isolated from porcine lung and recombinant porcine CGRP receptor expressed in HEK-293 cells with IC50 values of 350 ± 40 and 180 ± 15 nM, respectively (Fig. 3). SB-(+)-273779 had no significant affinity for the following receptors: recombinant human endothelin A and B and α1-adrenoreceptors (α1a, α1d) (in Chinese hamster ovary cells), calcitonin (T47D), and angiotensin II (rat adrenal cortex) when tested at 3 μM concentration with standard ligand binding assays. SB-(+)-273779 also did not inhibit forskolin or pituitary adenylyl cyclase-activating polypeptide-stimulated cAMP in SK-N-MC cells (Fig. 3). In contrast, the compound induced adrenomedullin binding and cAMP response with Kᵢ value of 1.5 μM in human calcitonin receptor-like receptor/receptor activity modifying protein-2 (RAMP2) in HEK-293 cells (data not shown). These data suggest that SB-(+)-273779 is a selective CGRP receptor antagonist. Due to lack of selective CGRP₂ receptor system (native or recombinant), we could not evaluate the affinity for SB-(+)-273779 in CGRP₂ receptor.

In addition to the activation of adenylyl cyclase, CGRP stimulated the release of intracellular [Ca²⁺]i in HEK-293 cells expressing recombinant human and porcine CGRP receptors with an EC₅₀ value of 1.7 ± 0.4 nM. The CGRP receptor antagonist, CGRP₈₋₉₇, by itself had no effect on [Ca²⁺]i release but inhibited the CGRP-stimulated [Ca²⁺]i release (10 nM). The nonpeptide antagonist, SB-(+)-273779, at 1.0 μM effectively blocked the CGRP-mediated cytosolic [Ca²⁺]i increase. The inhibitory effect was specific, since the compound has no effect on endothelin-1-mediated [Ca²⁺]i release in these cells (Fig. 6).

To investigate the effect of SB-(+)-273779 on CGRP-mediated carbohydrate metabolism, we used L6 skeletal muscle cells. L6 cells have functional CGRP receptors that are linked to stimulation of adenylyl cyclase, and this can be competitively antagonized by CGRP₈₋₉₇. Treatment of L6 cells with 1 μM insulin for 60 min stimulated [¹⁴C]2-deoxyglucose uptake by 2-fold. Inclusion of CGRP during the incubation period suppressed insulin-mediated deoxyglucose uptake. The maximum inhibition reached ~70% after incubation with 10 nM CGRP. Both CGRP₈₋₉₇ and SB-(+)-273779 have no direct effect on insulin-stimulated deoxyglucose uptake, but are able to reverse the CGRP-mediated suppression in a concentration-dependent manner (Fig. 7).

The effect of SB-273779 was also investigated in vasorelaxation experiments in vitro. Application of the solvent DMSO before CGRP, for 20 min, did not alter the vasorelaxing efficacy of the peptide. CGRP (3 nM) caused 21.6 ± 2.9% vasorelaxation in phenylephrine-contracted vessel rings. This effect was reduced by 76 ± 6% following application of the peptide antagonist, CGRP₈₋₉₇, at 1.0 μM (20 min before CGRP, Fig. 8). Pretreatment with SB-273779 from 0.1 to 1.0 μM caused a concentration-dependent inhibitory effect on the CGRP-mediated vasorelaxation. The inhibitory effect of SB-273779 reached 12 ± 9% (0.1 μM) and 35 ± 8% (0.3 μM). At higher concentration (1.0 μM), the effect of CGRP was reduced by 66 ± 4% (n = 5) (Fig. 9). The inhibitory effect of SB-(+)-273779 demonstrated a long duration of action. As shown in Fig. 9, initial recovery of vasorelaxing response to CGRP started to occur 40 min after removal of SB-(+)-273779 at 0.3 μM but not at 1.0 μM. In contrast, the effect of CGRP₈₋₉₇ was completely reversed after 20 min of washout.

We also investigated the effect of SB-(+)-273779 on the hypotensive effect mediated by bolus administration of CGRP. Intravenous administration of CGRP (100 or 300
pmol/kg) to the anesthetized rat pretreated with atropine and propranolol produced a dose-related reduction in arterial blood pressure (Fig. 10, A and B). Pretreatment with SB-(-)-273779 (10 min before the administration of CGRP) at 10 mg/kg antagonized the hypotensive effects of CGRP induced by both 100 (A) and 300 (B) pmol/kg (i.v.). No inhibitory effect was observed at a dose of 1 mg/kg of SB-(-)-273779. SB-273779 treatment had no effect on the transient hypotensive effects produced by sodium nitroprusside (30 ug/kg i.v.) administration (data not shown).

Discussion

In the present study, we have demonstrated that SB(-)-273779, a submicromolar-selective nonpeptide antagonist of the CGRP receptor, shows in vitro and in vivo activities. SB(-)-273779 is a selective inhibitor of [125I]CGRP binding to membranes isolated from human neuroblastoma cells (SK-N-MC) and HEK-293 cells expressing recombinant CGRP receptor with submicromolar (200–300 nM) affinity. The compound inhibited [125I]CGRP binding to membranes isolated from porcine or rat tissues by 40% only, even at 10 μM concentration. The specificity of SB(-)-273779 for CGRP receptors was demonstrated by its lack of activity (IC_{50} > 3 μM) in other radioligand binding assays, including recombinant human endothelin (A and B), α-adrenergic receptors as well as calcitonin receptors in T47D cells and rat angiotensin II receptors. To determine whether SB(-)-273779 interacts reversibly or irreversibly with CGRP receptors, [125I]CGRP binding in SK-N-MC cell membranes was determined in the presence and absence of SB(-)-273779. In the Scatchard analysis of the binding data, SB-273779 decreased the slope without affecting the intercept. Thus, the interaction of SB(-)-273779 with the CGRP receptor is reversible and competitive. Recently, Dodds et al. (2000) reported a novel CGRP receptor antagonist, BIBN 4096BS, with picomolar affinity for [125I]CGRP binding to SK-N-MC cell membranes. Based on [125I]CGRP radioligand binding studies performed in human (SK-N-MC) and rat (spleen) membranes, BIBN 4096BS has been characterized as a human-selective antagonist (Dodds et al., 2000). Thus, SB-273779 is further distin-
A. CGRP (100 pmol/kg, iv)

![Graph](image)

B. CGRP (300 pmol/kg, iv)

![Graph](image)

**Fig. 10.** Inhibition of CGRP-mediated hypotensive effect of SB-(+)-273779. CGRP was administered at time 0 (100 and 300 pmol/kg i.v. in A and B, respectively). SB273779 was administered 10 min before CGRP administration, and mean arterial pressure (MAP) was measured. *Differed significantly from the vehicle control (p < 0.05). n = 4 to 5 per group.

CGRP inhibited insulin-stimulated glucose uptake in a concentration-dependent manner. The maximal inhibition (70–80%) was observed at 100 nM CGRP. CGRP had no effect on basal glucose uptake in the cells. CGRP displaced [125I]CGRP binding from rat membranes. SB-(+)-273779 with the 1 nM concentrations produced a parallel shift in the CGRP concentration-response curve to the right without changing the maximum response suggesting competitive antagonism. However, prolonged treatment (>30 min) of SK-N-MC cells with SB-(+)-273779 (3 μM) followed by extensive washing revealed an apparent irreversible property on the compound. SB-(+)-273779 had no effect on forskolin or pituitary adenylate cyclase activating polypeptide-mediated adenylyl cyclase activity in SK-N-MC cell membranes. Additionally, SB-(+)-273779 inhibited CGRP-mediated intracellular [Ca^{2+}]i release from cloned CGRP1 receptor (human and porcine) expressed in HEK-293 cells.

In skeletal muscle, CGRP impairs glycogen synthesis and enhances glycolysis and lactate production. CGRP, like amylin, decreases basal and insulin-stimulated glycogen synthesis in muscle and stimulates lactate production from isolated soleus muscle (Cooper, 1994). Taken as a whole, these results indicate a potential role for CGRP for the pathophysiology of type 2 diabetes. Muscle has been suggested as an ideal tissue for glucose-uptake study because of its involvement in glucose utilization in vivo. However, the use of skeletal muscle tissue has several disadvantages for glucose-uptake studies (limited and inconsistent exposure of fibers to substrate). Alternatively, skeletal muscle cells in culture have proven to be a good model system for glucose-transport studies. Kreutter et al. (1989) have reported CGRP-mediated deoxyglucose uptake studies using L6 skeletal muscle cells. L6 cells have functional CGRP receptors that are linked to stimulation of adenylyl cyclase, and this can be competitively antagonized by CGRP-B.

CGRP inhibited insulin-stimulated glucose uptake in a concentration-dependent manner. The maximal inhibition (70–80%) was observed at 100 nM CGRP. CGRP had no effect on basal glucose uptake in the cells. CGRP blocked CGRP effect at a concentration of 300 nM. It was less potent in blocking CGRP effect on glucose uptake compared with its effect on CGRP-mediated adenylyl cyclase activation. SB-(+)-273779 also reversed the CGRP effect by >90% at 3 μM. As a negative control, several inactive compounds from benzanilide analogs were tested both in cyclase and binding assays (data not shown), which also had no effect on CGRP-mediated inhibition of glucose uptake.

The vasorelaxant activity of CGRP isoforms has been well documented within the mammalian vasculature. Indeed, such responses have been reported in a diverse range of vessels, including those isolated from rat (Mannan et al., 1995; Wisskirchen et al., 1998; Kawasaki et al., 1999), hamster (Hall and Brain, 1999), pig (Wisskirchen et al., 1999), and human (Thom et al., 1987).

To this end, the present study examined the ability of SB-273779 to attenuate the CGRP1-mediated vasodilator actions of CGRP in the isolated rat pulmonary artery. According to the ability of SB-(+)-273779 to attenuate CGRP-induced cAMP accumulation in SK-N-MC cells (IC_{50} 300 ± 18 nM), this benzanilide moiety inhibited the endothelial CGRP1 receptors responsible for transducing the relaxant responses induced by CGRP in the rat isolated pulmonary artery preconstricted with phenylephrine. Inhibition was concentration-dependent and was observed over a concentration range (0.1–1.0 μM) entirely consistent with a CGRP1-selective antagonist. As shown in Fig. 9, SB-273779 exhibited a poor reversibility of inhibitory action on CGRP-mediated relaxation in pulmonary artery. This effect of SB-273779 was also reported in experiments in vitro on the inhibition of CGRP-mediated adenylyl cyclase stimulation but was not observed with the peptide antagonist, CGRP-B. It may be suggested that the functional antagonistic action of SB-273779 is mediated via interaction with a binding site on the receptor, which is distinct from that of CGRP itself. This hypothesis is supported by the low potency of SB-273779 to displace [125I]CGRP binding from rat membranes.

In vivo, the intravenous administration of CGRP has been
shown to decrease arterial blood pressure, in a dose-dependent manner, by reducing total peripheral resistance (Lappe et al., 1987). CGRP-mediated reductions in arterial blood pressure are inhibited by the reversible peptide antagonist, CGRP$_{8-37}$. In the present study, we found that the intravenous administration of SB-(+)-273779 significantly reduced the dose-related hypertensive effects elicited by CGRP. The actions of SB-(+)-273779 were selective and did not alter direct nitrate-mediated smooth muscle relaxation and hypertension induced by the intravenous administration of sodium nitroprusside. In addition, the weak in vivo potency of SB-(+)-273779 precluded a more vigorous in vivo characterization. Doses greater than 10 (or 30 if any) mg/kg i.v. were not well tolerated, and a dose of 10 mg/kg i.v. did not inhibit significantly the CGRP-mediated hypertension. However, we did attempt to evaluate the role of CGRP in modulating insulin sensitivity in the Zucker rat, but we were not able to observe a consistent effect of CGRP on insulin sensitivity (CGRP$_{8-37}$, CGRP receptor antagonist failed to reduce insulin sensitivity in the model). Furthermore, we also attempted to evaluate the effect of SB-273779 on CGRP-induced hyperlactemia in normal rats. SB-273779 at 0.1 and 1.0 µmol/kg/min had no effect. At higher concentrations, the compound precipitated out. Preliminary studies also suggest that the pharmacokinetic profile of SB-273779 (poor oral bioavailability and short half-life of ~10 min) makes it unsuitable for use in chronic animal studies where repeated enteral administration is preferred.

In summary, SB-(+)-273779 is a selective CGRP receptor antagonist with functional activities. SB-273779 effectively inhibits CGRP-mediated vasorelaxation in vitro and in vivo. Although the apparent irreversibility of this interaction, at least in vitro, may not represent the optimal therapeutic profile for a CGRP antagonist, the nature of this interaction does not reduce the potential experimental utility of SB-273779. This antagonist, the first nonpeptide antagonist of “cross-species” (human, porcine, rat) CGRP receptors with in vitro antagonist activity at submicromolar concentration is likely to serve as a valuable pharmacological tool in the future. These data suggest that SB-(+)-273779, a prototype nonpeptide CGRP receptor antagonist, may help in elucidating specific CGRP receptor-mediated functions in complex biological systems, including in vivo pharmacological models.

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References