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Identification of the Atypical L-Type Ca^{2+} Channel Blocker Diltiazem and Its Metabolites As Ghrelin Receptor Agonists

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ABSTRACT

Using a high-throughput functional screen, the atypical L-type Ca^{2+} channel blocker diltiazem was discovered to be an agonist at the human ghrelin (GHSR1a) receptor. In cellular proliferation, Ca^{2+} mobilization, and bioluminescence resonance energy transfer (BRET-2) assays, diltiazem was a partial agonist at GHSR1a receptors, with 50 to 80% relative efficacy compared with the GHSR1a peptide agonist GHRP-6, and high nanomolar to low micromolar potency, depending upon the assay. Seven of the known primary metabolites of diltiazem were synthesized, and three of them (M_A, M_1, and M_2) were more efficacious and/or more potent than diltiazem at GHSR1a receptors, with a rank order of agonist activity of $M_2 > M_1 > M_A >$ diltiazem, whereas M_4 and M_6 metabolites displayed weak agonist activity, and the M_8 and M_9 metabolites were inactive. Binding affinities of diltiazem and these metabolites to GHSR1a receptors followed a similar rank order. In vivo tests showed that diltiazem and M_2 each stimulated growth hormone release in male Sprague-Dawley neonatal rats, although to a lesser degree than GHRP-6. Thus, diltiazem and chemical analogs of diltiazem represent a new class of GHSR1a receptor agonists. The possible contributions of GHSR1a receptor activation to the clinical actions of diltiazem are discussed in the context of the known beneficial cardiovascular effects of ghrelin.

Receptors that mediate the effects of growth hormone secretagogues (GHSs), including modified enkephalin peptides (Bowers et al., 1984) as well as small molecules (Smith et al., 1993), each capable of stimulating GH release, were only recently cloned (Howard et al., 1996). Two isoforms (A and B) were identified, but only growth hormone secretagogue receptor type 1a (GHSR1a) binds and responds to GHSs (Howard et al., 1996). Both isoforms are widely expressed, with the highest levels of GHSR1a found in the arcuate nucleus of the hypothalamus, and lower levels in cardiac tissue (Guan et al., 1997; Gnanapavan et al., 2002). Subsequently, a naturally occurring GHSR1a agonist was identified called ghrelin (Kojima et al., 1999).

A great deal of evidence exists linking ghrelin, GHSR1a receptors, food intake, and obesity in both animal models (Tschöp et al., 2000; Nakazato et al., 2001; Asakawa et al., 2003; Shearman et al., 2006) and humans (Cummings et al., 2001, 2002; Wren et al., 2001). However, ghrelin also regulates a variety of other endocrine and metabolic processes, including gut motility, energy homeostasis, cellular proliferation, and hormone production (for review, see van der Lely et al., 2004). It is noteworthy that ghrelin, and other GHS ligands produce a number of beneficial effects on cardiovas-

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ABBREVIATIONS: GHS, growth hormone secretagogue; GHSR1a, growth hormone secretagogue receptor type 1a; GHRP-6, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2; verapamil, 5-[*N*-(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride; nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; diltiazem, 5-(2-(dimethylamino)ethyl)-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydrobenzo[*b*][1,4]thiazepin-3-yl acetate; M_A, 2-(4-methoxyphenyl)-5-(2-(methylamino)ethyl)-4-oxo-2,3,4,5-tetrahydrobenzo[*b*][1,4]thiazepin-3-yl acetate; M_1, 5-(2-(dimethylamino)ethyl)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; M_2, 3-hydroxy-2-(4-methoxyphenyl)-5-(2-(methylamino)ethyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; M_4, 5-(2-(dimethylamino)ethyl)-3-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; M_6, 3-hydroxy-2-(4-hydroxyphenyl)-5-(2-(methylamino)ethyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; M_8, 5-(2-aminoethyl)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; M_9, 5-(2-aminoethyl)-3-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*][1,4]thiazepin-4(5*H*)-one; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HEK, human embryonic kidney; RGS, regulator of G-protein signaling; R-SAT, receptor selection and amplification technology; PBS, phosphate-buffered saline; GFP, green fluorescent protein; BRET, bioluminescence resonance energy transfer; GH, growth hormone.

cular function, including reductions in vascular resistance, vasodilation, increases in microvascular flow, increases in left ventricular ejection fraction and cardiac output, and cardioprotection in ischemia-reperfusion injury models (Nagaya et al., 2001a,b,c; Baldanzi et al., 2002; Bedendi et al., 2003; Benso et al., 2004; Cao et al., 2006). GHSR1a receptors are expressed in heart and vasculature, where they may mediate the beneficial effects of ghrelin and GHS ligands, although other receptors may also contribute (Baldanzi et al., 2002; Bedendi et al., 2003; Benso et al., 2004; Cao et al., 2006).

Diltiazem is an L-type Ca^{2+} channel blocker developed more than 30 years ago (Uchida, 1976) and used to treat patients suffering from angina pectoris, congestive heart failure, chronic obstructive pulmonary disease, and coronary artery spasm (Chaffman and Brogden, 1985; Glossmann and Striessnig, 1990). Diltiazem exerts its therapeutic effects by reducing vascular resistance, lowering blood pressure, increasing cardiac output, and lowering heart rate. In addition, diltiazem has been shown to provide cardioprotection against ischemia/reperfusion injury in rats (Takeo et al., 2004), similar effects as have been noted for ghrelin and other GHSs (discussed above). Diltiazem is extensively metabolized, and several of these metabolites reach significant concentrations in humans (Rovei et al., 1980; Sugihara et al., 1984; Sugawara et al., 1988; Molden et al., 2000). Several metabolites of diltiazem also show binding activity at Ca^{2+} channels, although all have lower affinity than diltiazem itself (Schoemaker et al., 1987).

To better understand the molecular basis for the clinical actions of drugs, we have been systematically screening and profiling collections of clinically used compounds for activity in functional assays using heterologously expressed G-protein coupled receptors. Using this strategy, diltiazem, and several metabolites of diltiazem were identified as novel GHSR1a agonists in a variety of in vitro and in vivo assays. The possible clinical significance of these findings is discussed.

Materials and Methods

Ligands. Ghrelin, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 (GHRP-6), D-Lys^3-GHRP-6, and substance P analog ([D-Arg1,D-Phe5,D-Trp7,9, Leu11]-substance P) were obtained from American Peptide (Sunnyvale, CA). Verapamil, methoxyverapamil, nifedipine, and diltiazem was purchased from Sigma-RBI. All diltiazem metabolites were synthesized at ACADIA (Olsson et al., 2006).

Cell Culture. NIH-3T3 cells (American Type Culture Collection, Manassas, VA) were incubated at 37°C in a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U/ml penicillin G, 50 U/ml streptomycin (Invitrogen), and 10% calf serum (Sigma, St. Louis, MO) or 25% Ultraculture synthetic supplement (Lonza Walkersville, Inc., Walkersville, MD). HEK 293 cells (American Type Culture Collection) were cultured similarly, except that 10% FCS was substituted for 10% calf serum.

Constructs. The GHSR1a receptor used in this study was cloned by polymerase chain reaction using oligonucleotides derived from the GenBank accession entry U60179. Regulator of G-protein signaling 1 (RGS1) was described previously (Burstein et al., 2005). Polymerase chain reactions were performed using Pfu Turbo (Stratagene, La Jolla, CA). All clones were sequence-verified before use.

Cellular Proliferation Assays. Cellular proliferation assays [also previously referred to as receptor selection and amplification technology (R-SAT) assays] were performed as described previously (Burstein et al., 2006) with the following modifications. In brief, cells were plated 1 day before transfection using 7×10^3 cells in 0.1 ml of media per well of a 96-well plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Cells were transiently transfected with 5 ng of receptor DNA, 2.5 ng of RGS1, and 30 ng of pSI-β-galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The use of RGS1 was found to suppress the constitutive activity of GHSR1a and to improve agonist responses in this functional assay (Burstein et al., 2005). One day after transfection, the medium was changed and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Cambrex Bio Science Walkersville, Inc.) instead of calf serum to a final volume of 200 μl per well. After 5 days in culture, β-galactosidase levels were measured essentially as described previously (Burstein et al., 2005). Cells were rinsed with phosphate-buffered saline (PBS), pH 7.4, before the addition of 200 μl of PBS supplemented with 3.5 mM *O*-nitrophenyl-β-D-galactopyranoside and 0.5% Nonidet P-40 (both from Sigma). After incubation (2–4 h), the plates were read at 420 nm on a plate reader (EL 310; Bio-Tek Instruments, Winooski, VT; or Molecular Devices, Sunnyvale, CA).

Ca^{2+} Mobilization Assays. Intracellular changes in calcium concentrations as a result of activation of GHSR1a receptors were detected using the calcium binding bioluminescence protein aequorin, which was expressed as part of a tripartite chimeric protein, MT-GFP-AEQ, as described previously (Burstein et al., 2006).

Bioluminescence Resonance Energy Transfer Assays. BRET-2 assays were performed as described previously (Schiffer et al., 2007) with the following modifications: HEK293T cells cultured in 10-cm^2 plates were transiently transfected with plasmid DNAs expressing a bioluminescence donor (1 μg of plasmid DNA expressing GHSR1a carboxyl-terminally tagged with *Renilla reniformis* luciferase) and a fluorescence acceptor (40 μg of plasmid DNA expressing β-arrestin-2 amino-terminally tagged with GFP2). Two days after transfection, cells were harvested and resuspended in PBS, pH 7.5, with glucose and sodium pyruvate to a concentration of 2 to 4×10^6 cells/ml dependent on transfection efficiency. BRET-2 signals were calculated as the ratio between the *R. reniformis* luciferase emission and the GFP2 emission corrected by the background emissions of nontransfected cells.

Binding Assays. Dishes (15 cm^2) were seeded with 4 million cells in 16 ml of 10% FCS/1% penicillin/streptomycin/L-glutamine/DMEM for transfection the next day. Plasmid DNA containing the GHSR1a receptor (12.5 μg/dish) in 0.675 ml of DMEM was transfected into the cells by mixing with 180 μl of PolyFect, mixing in 2.25 ml of 10% FCS/DMEM 15 min later, and transferring the mixture into the dish. At 16 to 18 h after transfection, medium was replaced with 25 ml of fresh 10% FCS/1% penicillin/streptomycin/L-glutamine/DMEM to each dish for another 18 to 20 h. At approximately 48 h after transfection, cells were harvested in ice-cold membrane buffer (20 mM HEPES, 6 mM $MgCl_2$, and 1 mM EDTA, pH adjusted to 7.2) using a cell scraper and pelleted by centrifugation. Pelleted cells were added to a nitrogen cavitation chamber, and 900 bar (90 MPa) of pressure was applied for 30 min. The pressure was slowly released the cavitated cells collected in 50-ml Falcon tubes. The tubes were centrifuged at 1000 rpm, 4°C for 10 min, and the supernatant was collected. This centrifugation and collection was repeated twice more until the supernatant was free of precipitate (membranes were still in suspension). The supernatant was poured into a 50-ml centrifuge tube and centrifuged at 10,000 rpm, 4°C for 20 min. The supernatant was discarded and the pellet resuspended in 750 μl of membrane binding buffer using a chilled 1-ml syringe with 25G, 5/8-inch needle to resuspend membranes. The protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The protein concentration was adjusted to 5 mg/ml, and aliquots were snap-frozen and stored at −80°C until use. Membranes were thawed rapidly, diluted with binding buffer (25 mM HEPES + 5 mM $MgCl_2$,

1 mM CaCl, 2.5 mM EDTA, and 0.2% bovine serum albumin) to a protein concentration of 0.8 μg/30 μl and placed on ice. Ninety-six-well plates (U-bottom wells) were prepared with serial dilutions (eight doses, 40 μl/well) of the test compounds. Membranes (30 μl/well) were then added, and incubated with test ligands for 30 min at room temperature with shaking. ^{125}I-Ghrelin (30 μl; 0.053 nM) was then added to each well, and the plates were incubated for another 2.5 h with shaking. Binding was terminated by filtration through GF/B filters (presoaked with 0.1% polyethylenimine) with a 96-well harvester (Brandel Inc., Gaithersburg, MD). The filters were washed with ice-cold binding buffer (150 ml/plate) and allowed to air-dry for 30 min. MicroScint-20 cocktail (50 μl) was added to each dried well, and the plates were sealed and counted for 2 min/well using a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Data Analysis. Concentration-response graphs for all functional assays were plotted, and EC_{50} values were determined by nonlinear regression analysis using Prism software (version 4.0; GraphPad Software, San Diego, CA) according to the equation: $Y = Bottom + (Top - Bottom)/(1 + 10^{Log\ EC_{50} - X})$, where X is the logarithm of concentration and Y is the response. Y starts at *Bottom* and goes to *Top* with a sigmoid shape. Allowing the Hill coefficient to vary did not significantly change the fits of the curves, and thus the Hill coefficient was constrained to unity.

Growth Hormone Releasing Assays. Male Sprague-Dawley neonates (~35 g; 16 days old) were housed with free access to rat chow/water at 6 animals per cage for at least 2 days before use. Drugs were dosed i.p. (~9:30 AM) in 5% DMSO/95% polyethylene glycol 400 vehicle. Before sacrifice, rats were anesthetized with isoflurane (Aerrane; Baxter, McGaw Park, IL). Trunk blood samples were collected 15 min after i.p. injection. Plasma was isolated by centrifugation at 3000 rpm for 15 min then stored at −80°C until use. Rat GH levels were measured using rat growth hormone enzyme immunoassay kits (SPI-BIO, Montigny le Bretonneux, France) according to the manufacturer's instructions.

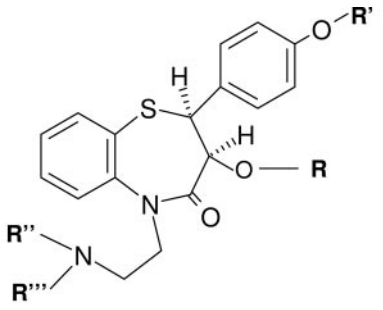
Results

We have developed a high-throughput cellular proliferation assay that is compatible with most G-protein-coupled receptors and that detects constitutive receptor activity with high sensitivity (R-SAT; Burstein et al., 2005; 2006). To identify novel small molecule ligands, R-SAT was used to screen the human GHSR1a receptor against a collection of more than 200,000 compounds. The compound library included products of combinatorial chemical synthesis, medicinal chemistry synthesis, and more than 2000 clinically used drugs. A large number of novel agonists and inverse agonists at the GHSR1a receptor were identified, most of which will not be described further here.

Among the "hits " identified was the L-type Ca^{2+} blocker diltiazem (Fig. 1). Full concentration-response experiments confirmed that diltiazem is a partial agonist at GHSR1a, with approximately 70% efficacy compared with GHRP-6, and a potency of 400 nM (Fig. 2, Table 1). Diltiazem-induced responses in cellular proliferation were completely suppressed by the ghrelin-receptor inverse agonist substance P analog, verifying that diltiazem was mediating responses through activation of GHSR1a (data not shown). We observed no significant functional response of GHSR1a receptors to other Ca^{2+} channel blockers verapamil, its methoxy analog, or nifedipine (Table 1).

Diltiazem is extensively metabolized (Rovei et al., 1980; Sugihara et al., 1984; Sugawara et al., 1988; Molden et al., 2000; see Fig. 1), and several of its metabolites also possess Ca^{2+} channel-blocking activity (Schoemaker et al., 1987). Seven of the primary metabolites of diltiazem were synthesized and tested for activity at GHSR1a. Several of the diltiazem metabolites were also agonists at GHSR1a. The M_A, M_1, and M_2 metabolites were significantly more efficacious, and M_1 and M_2 were more potent than diltiazem itself, with pEC_{50} values of approximately 200 nM (M_1 and M_2) and efficacies of 89% to more than 100%, respectively (Fig. 2, Table 1). The M_4 and M_6 metabolites displayed weak agonist activity at GHSR1a. The M_8 and M_9 metabolites did not display agonist activity at GHSR1a and were unable to block GHRP-6 induced responses, indicating that they did not act as antagonists at GHSR1a either (data not shown). The potencies of GHRP-6, ghrelin, and hexarelin in the cellular proliferation assay were similar to values reported previously (Holst et al., 2005); however, ghrelin displayed surprisingly low efficacy in the cellular proliferation assay.

GHSR1a receptors couple the G-protein G_q to release intracellular Ca^{2+} (Howard et al., 1996), and like virtually all G-protein-coupled receptors, agonist-activated GHSR1a receptors recruit and bind β-arrestin (Holst et al., 2005). Therefore, to verify the results obtained using the cellular proliferation assay, diltiazem and the active metabolites were tested for agonist activity at GHSR1a in Ca^{2+} mobilization assays and bioluminescence resonance energy transfer (BRET-2) assays in which luciferase-tagged receptors (GHSR1a-luc) were cotransfected with GFP-tagged β-arrestin-2 (see *Materials and Methods*). Separate experiments verified that activity of the GHSR1a receptor in the cellular proliferation assay was not significantly altered by the presence of the luciferase tag (data not shown). The pEC_{50} values of GHRP-6 in the Ca^{2+} and BRET-2 assays were 9.1 and 8.4, respectively, in good agreement with its potency in the cellular proliferation assay (see Table 1). Similar to the cellular



Ligand	R	R'	R''	R'''
DTZ	-$COCH_3$	CH_3	CH_3	CH_3
MA	-$COCH_3$	CH_3	H	CH_3
M1	H	CH_3	CH_3	CH_3
M2	H	CH_3	H	CH_3
M4	H	H	CH_3	CH_3
M6	H	H	H	CH_3
M8	H	CH_3	H	H
M9	H	H	H	H

Sagarawa et al. 1988

Fig. 1. Diltiazem and its metabolites. [Adapted from Sugawara Y, Ohashi M, Nakamura S, Usuki S, Suzuki T, Ito Y, Kume T, Harigaya S, Nakao A, Gaino M, et al. (1988) Metabolism of diltiazem. I. Structures of new acidic and basic metabolites in rat, dog and man. *J Pharmacobiodyn* **11:**211–223. Copyright © 1988 Pharmaceutical Society of Japan. Used with permission.

proliferation assay, and as seen previously in BRET-2 assays (Holst et al., 2005), ghrelin was a partial agonist in BRET-2, although it was essentially a full agonist in Ca^{2+} mobilization assays.

Testing of diltiazem and its active metabolites in each of these functional assays yielded similar results compared with the cellular proliferation assay (Table 1). In the Ca^{2+} mobilization assay, diltiazem and all the tested diltiazem metabolites were partial agonists with varying degrees of efficacy, ranging from approximately 54% (diltiazem) to approximately 80% (M_2) (Fig. 3, Table 1). Potencies ranged from a pEC_{50} of 5.8 for diltiazem to 6.7 for M_2. Likewise, in BRET-2 assays, diltiazem and the diltiazem metabolites M_A, M_1, and M_2 were each partial agonists; again, both M_1 and M_2 were significantly more potent than diltiazem or M_A (Fig. 4, Table 1). Thus the rank order of potency across all three functional assays was $M_2 \geq M_1 > M_A \geq$ diltiazem (Table 1).

To directly verify that diltiazem and its primary metabolites are human GHSR1a receptor ligands, radioligand-binding assays were carried out using cell membranes expressing human GHSR1a receptors, using ^{125}I-ghrelin as the radioligand. Diltiazem and its primary metabolites M_A, M_1, and M_2 each completely displaced ^{125}I-ghrelin with a rank order of potency similar to that seen in the functional assays (Table 2).

A characteristic of GHSR1a agonists such as GHRP-6 is the ability to stimulate the release of GH (Bowers et al., 1984). Diltiazem and the M_2 metabolite of diltiazem were administered to male Sprague-Dawley neonatal rats, and plasma levels of growth hormone were assessed. Both diltiazem and M_2 were able to stimulate increases in plasma GH levels, although only the increases in response to diltiazem were statistically significant (Fig. 5, Table 3). The diltiazem-induced increases were approximately 40% of the maximal effect of GHRP-6

Discussion

We have used a high-throughput functional screen to identify the L-type Ca^{2+} channel blocker diltiazem as a human GHSR1a receptor agonist. Using three different functional assays, each measuring distinct functional responses (Ca^{2+} mobilization, cellular proliferation, and β-arrestin recruitment), we showed that diltiazem is a partial agonist of the human GHSR1a receptor. In addition, we synthesized and tested seven known metabolites of diltiazem, including all of

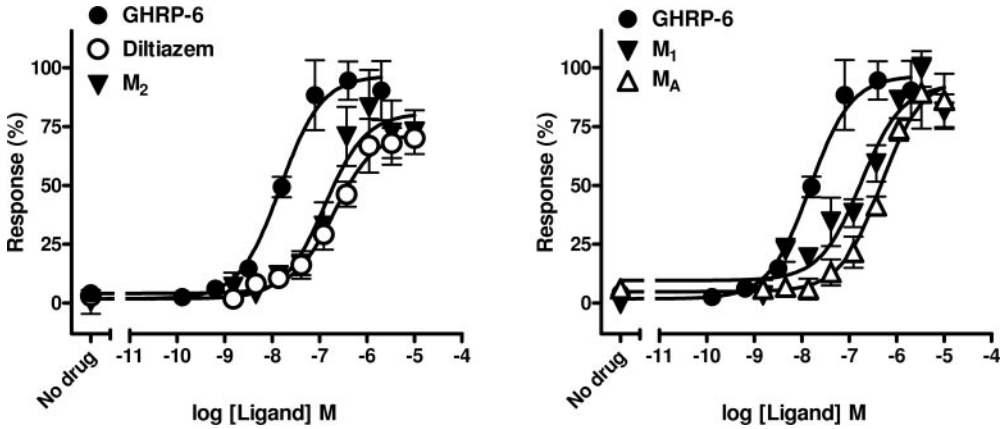


Fig. 2. Diltiazem pharmacology—cellular proliferation. The human GHSR1a receptor was transfected into NIH3T3 cells and analyzed for proliferative responses as described in the methods. Proliferative responses to the indicated concentrations of GHRP-6, diltiazem, and M_A (A) and ghrelin, M_1, and M_2 (B) were measured as described under *Materials and Methods*. Data points are the means of two determinations ± S.D.

TABLE 1

Functional profile of Diltiazem and metabolites at the human ghrelin receptor

Functional assays were performed as described under *Materials and Methods*. Values represent the means ± S.E.M.

Ligand	Cell Proliferation		Ca^{2+} Mobilization		BRET	
	pEC_{50}	Efficacy	pEC_{50}	Efficacy	pEC_{50}	Efficacy
	nM	%	*nM*	%	*nM*	%
GHRP-6	8.4 ± 0.0	100 ± 1	9.1 ± 0.1	100 ± 0	8.4 ± 0.1	100 ± 0
Ghrelin	8.9 ± 0.3	35 ± 2	8.4 ± 0.1	96 ± 1	8.6 ± 0.1	83 ± 2
Hexarelin	8.4 ± 0.0	107 ± 8	N.D.		8.4 ± 0.1	91 ± 10
Diltiazem	6.4 ± 0.1	76 ± 3	5.8 ± 0.1	54 ± 3	5.0 ± 0.2	49 ± 8
M_A	6.0 ± 0.3	98 ± 5	5.9 ± 0.1	69 ± 6	5.1 ± 0.2	77 ± 3
M_1	6.6 ± 0.1	89 ± 4	6.7 ± 0.1	71 ± 3	6.1 ± 0.1	60 ± 4
M_2	6.6 ± 0.1	107 ± 4	6.7 ± 0.1	81 ± 4	6.0 ± 0.1	70 ± 1
M_4	4.9 ± 0.2	40 ± 6	N.D.		5.1 ± 0.0	75 ± 5
M_6	4.4 ± 0.4	65 ± 5	N.D.		N.D.	
M_8		12	N.D.		N.D.	
M_9		7	N.D.		N.D.	
Verapamil		N.R.	N.D.		N.D.	
Me-Verapamil		N.R.	N.D.		N.D.	
Nifedipine		N.R.	N.D.		N.D.	

N.D., not done; N.R., no response; Me-Verapamil, methoxyverapamil.

the most abundant ones. Functional profiling revealed that many of these compounds were also agonists at human GHSR1a receptors. Two of the most abundant diltiazem metabolites, M_1 and M_2, were each more potent, more efficacious GHSR1a receptor agonists than diltiazem itself. The rank order of activity for diltiazem and its metabolites was consistently $M_2 \geq M_1 > M_A \geq$ diltiazem across all functional and binding assays (Tables 1 and 2). In contrast, the rank order of potency of GHRP-6 and ghrelin was ghrelin > GHRP-6 in the cellular proliferation assay, in BRET-2, and in binding assays but was reversed in Ca^{2+} mobilization assays, similar to previous observations (Holst et al., 2005). Ghrelin was a partial agonist compared with GHRP-6 in cellular proliferation and BRET-2 assays, in agreement with previous observations that ghrelin is a partial agonist in some functional assays (Holst et al., 2005).

Further tests revealed that diltiazem, and M_2, the most potent and efficacious GHSR1a-receptor agonist of the diltiazem metabolites, acted as GHSR1a agonists in vivo. Both diltiazem and M_2 stimulated GH-release in neonatal rats. Compared with GHRP-6, the maximal effects of diltiazem and M_2 were each approximately 35 to 40% in GH release assays, consistent with their partial agonist profiles in vitro.

Diltiazem is used to treat cardiovascular disorders such as angina pectoris, congestive heart failure, chronic obstructive pulmonary disease, hypertension, and coronary artery spasm (Chaffman and Brogden, 1985; Glossman and Striessnig, 1990). Although diltiazem is thought to mediate these effects through blockade of L-type Ca^{2+} channels, it is interesting to speculate whether GHSR1a receptors contribute to some of the therapeutic benefits of diltiazem therapy. Peptidyl GHS, nonpeptidyl GHS, and ghrelin itself are all known to produce a number of beneficial cardiovascular effects, including reductions in vascular resistance, vasodilation, increases in microvascular flow, increases in left ventricular ejection fraction and cardiac output, and cardioprotection against ischemia and cellular apoptosis in ischemia-reperfusion injury paradigms (Nagaya et al., 2001a,b,c; Baldanzi et al., 2002; Bedendi et al., 2003; Benso et al., 2004; Cao et al., 2006), effects similar to those that occur with diltiazem administration. Diltiazem has been shown to provide cardioprotection against ischemia/reperfusion injury in rats, and it has been hypothesized that these protective effects are mediated through preservation of mitochondrial function by attenuating Na^+ overload through Na^+ channels (Takeo et al., 2004). The cardioprotective effects of diltiazem in such models may be mediated, in part, through activation of GHSR1a receptors, which have antiapoptotic effects in cardiomyocytes (Baldanzi et al., 2002). Besides the hypothalamus, GHSR1a receptors are widely distributed and are expressed throughout the myocardium and vasculature, including ventricle and aorta (Gnanapavan et al., 2002, Guan et al., 1997; Cao et al., 2006), where they could mediate some of the cardiovascular effects of GHS, ghrelin, and possibly diltiazem. It would be interesting to evaluate the possible contributions of GHSR1a

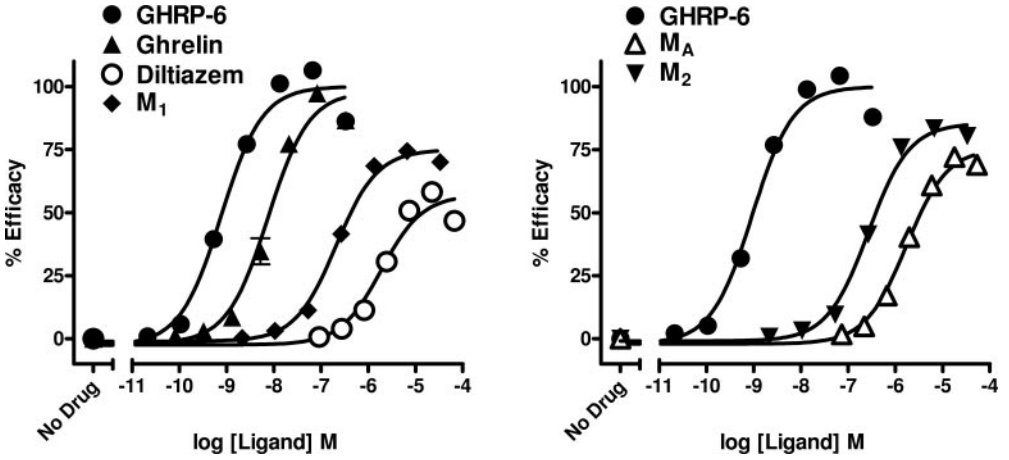


Fig. 3. Diltiazem pharmacology—Ca^{2+} mobilization. The human GHSR1a receptor was transfected with coelenterazine into HEK293T cells. Cells were harvested and analyzed for Ca^{2+} release in the presence of the indicated ligands as described under *Materials and Methods*. Data points are the means of two determinations ± S.D. Ligand responses to GHSR1a were normalized to the response to GHRP-6, which was 40 relative luminescence units (RLUs).

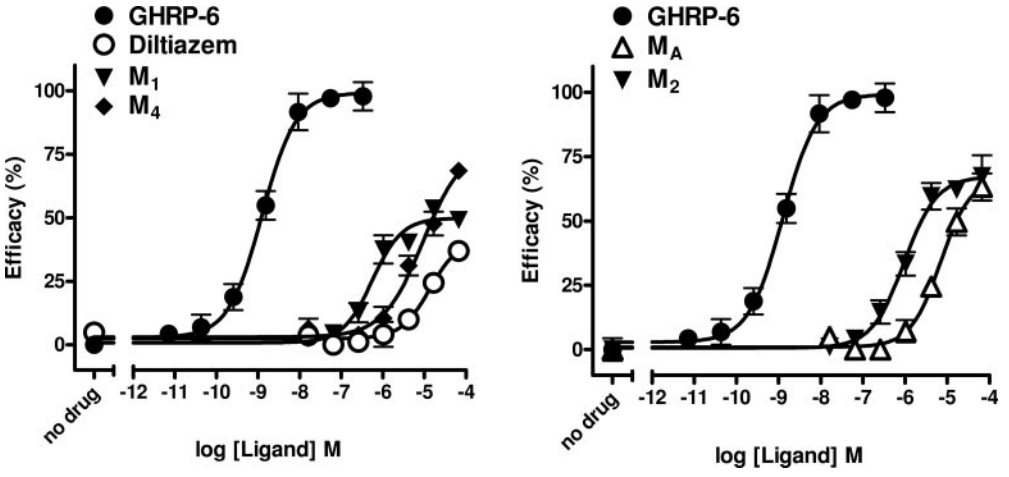


Fig. 4. Diltiazem pharmacology—BRET-2. Human GHSR1a receptor fused to *R. reniformis* luciferase on its C terminus was cotransfected with human β-arrestin-2 fused to GFP on its C terminus into HEK293T cells. Cells were harvested and analyzed for BRET-2 activity in the presence of the indicated ligands as described under *Materials and Methods*. Data points are the means of two determinations ± S.D. Ligand responses to GHSR1a were normalized to their responses to GHRP-6.

receptors to the cardioprotective effects of diltiazem using GHSR1a-selective antagonists, or GHSR1a-null mice.

Diltiazem has not been reported to elevate GH or cause weight gain clinically (McGraw et al., 1982), and diltiazem was reported to inhibit food intake in Sprague-Dawley rats (de Beaurepaire and Freed, 1989; Amer and Maher, 2005). We have not observed that diltiazem inhibits food intake and may even modestly stimulate food intake in rats (J.-N. Ma and E. S. Burstein, unpublished observations). The apparent lack of effect of long-term diltiazem administration on GH release or food intake could be interpreted as a lack of support for the role of ghrelin agonists in these effects; however it is probably more likely to be due to the primary effects of diltiazem (i.e., blockade of Ca^{2+} influx), which may impair GH release (Lussier et al., 1988), and/or inadequate drug exposure (Rovei et al., 1980; Sugihara et al., 1984; Chaffman and Brogden, 1985), given its potency for activating GHSR1a receptors (current study) relative to its affinity for L-type Ca^{2+} channels (Glossmann and Striessnig, 1990; Zobrist and Mecca, 1990), and is also consistent with our observations that diltiazem is a partial agonist at GHSR1a receptors. Diltiazem peak plasma concentrations as high as 152 ng/ml were reported in healthy adult male volunteers (Zelis and Kinney, 1982), which, depending upon the assay, is comparable with or significantly lower than the pEC_{50} values reported here (Table 1). These plasma concentrations are probably insufficient to mediate significant effects on GH-release or food intake given that these are peak rather than sustained concentrations and do not take into account other factors that would reduce the free concentration of diltiazem at the target tissues, such as protein binding (Yamano et al., 2000).

Diltiazem is extensively metabolized (Rovei et al., 1980; Sugihara et al., 1984; Sugawara et al., 1988; Molden et al., 2000), and some of its metabolites, notably desacetyl diltiazem (M_1), reach plasma concentrations approaching 20 to 30% that of diltiazem itself in humans (Rovei et al., 1980; Sugihara et al., 1984). All of the metabolites studied here displace [^{3}H]diltiazem binding, allosterically enhance [^{3}H]nitrendipine binding, and block Ca^{2+}-dependent contractions of isolated rat portal veins, but all at lower affinity than diltiazem itself (Schoemaker et al., 1987). The M_2 metabolite, which we found to be significantly more potent and efficacious as a GHSR1a agonist than diltiazem, had approximately 10-fold lower activity in L-type Ca^{2+} channel binding and functional assays than diltiazem (Schoemaker et al., 1987). Thus, the structure-activity relationships for interactions of diltiazem and its metabolites with L-type Ca^{2+} channels and GHSR1a receptors diverge.

Given the results presented above, medicinal chemistry efforts using the diltiazem scaffold may yield potent GHSR1a agonists. Indeed, the diltiazem scaffold is amenable to medicinal chemistry, as shown previously (Floyd et al., 1992). The divergence between GHSR1a activity and L-type Ca^{2+} channel binding activity (Schoemaker et al., 1987) observed in the structural analogs tested here demonstrates the potential for developing potent GHSR1a agonists lacking L-type Ca^{2+} channel blocking activity from the diltiazem scaffold.

TABLE 2

Binding affinities of diltiazem and metabolites at the human ghrelin receptor

Functional assays were performed as described under *Materials and Methods*. Values represent the means ± S.E.M.

Ligand	pK_i
Ghrelin	10.4 ± 0.3
GHRP-6	8.6 ± 0.0
Hexarelin	8.5 ± 0.0
Diltiazem	5.5 ± 0.1
M_A	5.2 ± 0.1
M_1	6.1 ± 0.2
M_2	6.3 ± 0.2
M_4	5.2 ± 0.1
M_6	4.9 ± 0.0

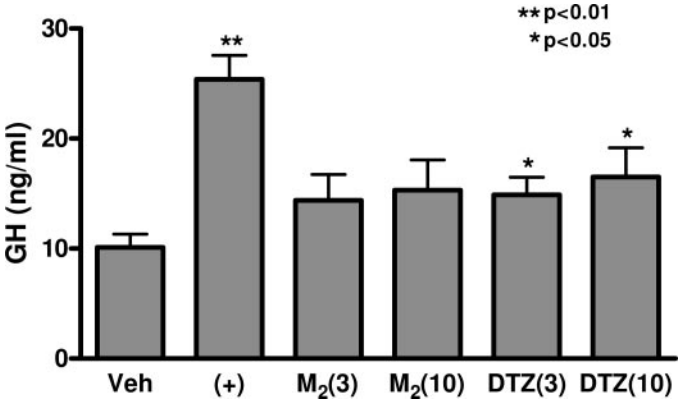


Fig. 5. Growth hormone releasing activity of diltiazem and its M_2 metabolite. Freely moving, freely feeding male Sprague-Dawley rats, approximately 100 to 120 g, were injected i.p. with the indicated doses (milligrams per kilogram) of either diltiazem or the M_2 metabolite of diltiazem. Plasma levels of growth hormone were measured by rat growth hormone enzyme immunoassay kits as described under *Materials and Methods*. Data are the means of nine to thirty-three determinations ± S.E.M. (+) indicates 0.5 mg/kg GHRP-6.

TABLE 3

Growth hormone release in rats

Growth hormone releasing assays were performed as described under *Materials and Methods*. Neonatal rats were dosed i.p. with indicated amounts of drugs. Fifteen minutes after dosing, animals were sacrificed, and blood was harvested and analyzed for growth hormone levels using a rat growth hormone enzyme immunoassay kit as described under *Materials and Methods*. Data represent means ± S.E.M.

Compound	Dose	GH	*t* Test
	mg/kg	*ng/ml*	
Vehicle		10.1 ± 1.2	
GHRP-6	0.25	25.4 ± 2.2	$P<0.01$
M_2	3	14.4 ± 2.3	$P=0.10$
M_2	10	15.3 ± 2.8	$P=0.08$
Diltiazem	3	14.9 ± 1.6	$P<0.05$
Diltiazem	10	16.5 ± 2.7	$P<0.05$

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