British Journal of Pharmacology (2006) 147, 73–82
www.nature.com/bjp

Characterization of the Mas-related gene family: structural and functional conservation of human and rhesus MrgX receptors

*,[1]Ethan S. Burstein, [1]Thomas R. Ott, [1]Michele Feddock, [1]Jian-Nong Ma, [1]Steve Fuhs, [1]Steven Wong, [1]Hans H. Schiffer, [1]Mark R. Brann & [1]Norman R. Nash

[1]Acadia Pharmaceuticals Inc., 3911 Sorrento Valley Boulevard, San Diego, CA 92121, U.S.A.

1 Recently, a large family of G-protein-coupled receptors called Mas-related genes (Mrgs), which is selectively expressed in small-diameter sensory neurons of dorsal root ganglia, was described. A subgroup of human Mrg receptors (MrgX1–X4) is not found in rodents and this has hampered efforts to define the physiological roles of these receptors.

2 MrgX receptors were cloned from rhesus monkey and functionally characterized alongside their human orthologs. Most of the human and rhesus MrgX receptors displayed high constitutive activity in a cellular proliferation assay. Proliferative responses mediated by human or rhesus MrgX1, or rhesus MrgX2 were partially blocked by pertussis toxin (PTX). Proliferative responses mediated by rhesus MrgX3 and both human and rhesus MrgX4 were PTX insensitive. These results indicate that human and rhesus MrgX1 and MrgX2 receptors activate both Gq- and Gi-regulated pathways, while MrgX3 and MrgX4 receptors primarily stimulate Gq-regulated pathways.

3 Peptides known to activate human MrgX1 and MrgX2 receptors activated the corresponding rhesus receptors in cellular proliferation assays, Ca^{2+}-mobilization assays, and GTP-γS-binding assays. Cortistatin-14 was selective for human and rhesus MrgX2 receptors over human and rhesus MrgX1 receptors. BAM22 and related peptides strongly activated human MrgX1 receptors, but weakly activated rhesus MrgX1, human MrgX2, and rhesus MrgX2 receptors.

4 These data suggest that the rhesus monkey may be a suitable animal model for exploring the physiological roles of the MrgX receptors.
British Journal of Pharmacology (2006) **147,** 73–82. doi:10.1038/sj.bjp.0706448;
published online 14 November 2005

Keywords: Nociception; dorsal root ganglia; mas; G-protein-coupled receptor; Bam22; cortistatin-14

Abbreviations: BAM22, bovine adrenal medulla peptide 22; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; DRR, dorsal root receptor; EDTA, ethylenediamine tetraacetate; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; Mrg, Mas-related gene; PTX, pertussis toxin; SNSR, sensory neuron-specific receptor; STIA, somatostatin tumor-inhibiting analog

Introduction

The G-protein-coupled receptor (GPCR) superfamily is the most exploited gene family for drug discovery; yet, over 50% of the GPCR family remains classified as 'orphan receptors', receptors whose functions and regulatory ligands remain unknown. Given that GPCRs are one of the most targeted gene families for therapeutic intervention, understanding the physiological roles of these genes is of great importance.

Recently, the identification of a large family of GPCRs that are related to the MAS oncogene (Young *et al*., 1986), called Mas-related genes (Mrgs), was reported (Dong *et al*., 2001). These genes, also known as the sensory neuron-specific receptors (SNSRs) or dorsal root receptors (DRRs), comprise a large family of over 50 rodent and human orphan GPCRs. The restricted expression of many members of the Mrg family to sensory neurons of the dorsal root ganglion (DRG) suggests that these receptors may play a role in nociception; however, this has not yet been conclusively established. These genes were first identified by subtracting cDNAs from neonatal murine wild-type and Neurogenin1 ($Ngn1^{-/-}$) knockout DRG. $Ngn^{-/-}$ mice do not generate sensory neurons (Ma *et al*., 1999). In humans, the MrgX subset of Mrg receptors is selectively expressed in DRG (Lembo *et al*., 2002). Expression of a subset of these genes is primarily restricted to small-diameter sensory neurons in both rodents and in humans (Dong *et al*., 2001; Lembo *et al*., 2002; Robas *et al*., 2003), further implicating these receptors in nociception. Interestingly, the DRG-specific members of this family show discrete patterns of expression that appear to be only partially overlapping within the sensory neurons, implying that these receptors may each play unique roles in pain sensation.

A major limitation in developing animal models for understanding the function of the MrgX receptors is that MrgX orthologs do not exist in rodents. Indeed, the genomic organization of Mrg receptors varies quite dramatically even among highly related species. For example, the mouse genome contains 22 MrgA genes and 14 MrgC genes, whereas rat contains only one each, a difference that is thought to exist because of an atypical expansion of this receptor family (Zylka *et al*., 2003). The expression patterns of Mrg receptors

*Author for correspondence; E-mail: eburstein@acadia-pharm.com

are similar between mouse and rat, with DRG-specific receptors expressed in neurons which are IB4 positive, and express the glial cell line-derived neurotrophic factor (GDNF) receptor c-Ret (Snider & McMahon, 1998; Julius & Basbaum, 2001; Zylka *et al*., 2003). Despite these similarities, MrgD receptors in rat are coexpressed with the capsaicin receptor VR1, whereas in mouse they are not, and in rat all Mrg-expressing neurons coexpress the purinergic receptor P2X3, whereas in mouse only MrgD is coexpressed with P2X3 (Zhang *et al*., 2005). However, no orthologs of human MrgX receptors exist in any of the rodent species examined to date. Although limited functional characterization of this family has been described (Dong *et al*., 2001; Bender *et al*., 2002; Han *et al*., 2002; Lembo *et al*., 2002; Robas *et al*., 2003), the functional properties and physiological functions of these genes remain largely unknown. It is assumed that rodent Mrg receptors expressed in DRG probably perform similar functions as human receptors expressed in DRG. However, given the differences between human and rodent receptors, and given that each receptor subtype may perform specific functions, extrapolating results from experiments with rodents may not accurately predict the functions of human receptors.

Recently, MrgX receptors have been shown to be expressed in macaque (Zhang *et al*., 2005). As rhesus monkeys are a commonly used animal model for experimental biology and drug discovery, we cloned and pharmacologically analyzed rhesus monkey MrgX receptor counterparts for each of the known human MrgX receptors, and found that they are functionally as well as structurally similar to the known human MrgX receptors. These results indicate that rhesus monkeys may be useful for exploring the functions of these receptors.

Methods

NIH-3T3 cells were from ATCC (Manassas, VA, U.S.A.) CRL 1658. HEK293 cells were from ATCC (Manassas, VA, U.S.A.) CRL 1573. *O*-nitrophenyl-β-D-galactopyranoside and nonidet P-40 were from Sigma (St Louis, MO, U.S.A.). Tissue culture medium used was Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA, U.S.A.) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U ml^{-1} penicillin G, 50 U ml^{-1} streptomycin (Invitrogen-Gibco, Carlsbad, CA, U.S.A.) and 10% calf serum (Sigma, St Louis, MO, U.S.A.) or 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD, U.S.A.). 96-well, six-well, and 15 cm^2 tissue culture dishes were from Falcon. Hank's balanced salt solution without magnesium chloride, magnesium sulfate, and calcium chloride and trypsin–EDTA were from Invitrogen-Gibco (Carlsbad, CA, U.S.A.). BAM22, somatostatin tumor-inhibiting analog (STIA, full peptide name DNal-Cys-Tyr-DTrp-Lys-Val-Cys-Thr-NH_2) and cortistatin-14 were obtained from American Peptide (Sunnyvale, CA, U.S.A.). ([8–22]BAM22 was obtained from Tocris (Ellisvilee, MO, U.S.A.).

Cell culture

NIH-3T3 cells were incubated at 37°C in a humidified atmosphere (5% CO_2) in supplemented DMEM. HEK293 cells were cultured similarly, except that 10% fetal calf serum was substituted for 10% calf serum.

Constructs

The MrgX receptors used in this study were cloned by polymerase chain reaction. Primers were designed to anneal on either side of the coding sequence of the human MrgX genes or in the untranslated regions of potential MrgX of *Macaca mulatta* (sequences were obtained from the NCBI trace archives: http://www.ncbi.nlm.nih.gov/Traces/trace.cgi). PCRs with these primers were performed on genomic DNA obtained from blood using Pfu Turbo (Stratagene, La Jolla, CA, U.S.A.) to obtain full-length human MrgX1, MrgX2, MrgX3, and MrgX4, as well as the rhesus orthologs. The human M1 and M5 muscarinic acetylcholine receptors have been described (Bonner *et al*., 1987; 1988). Ras/rap1B (AA) has been described (Weissman *et al*., 2004) and was constructed by ligating PCR-amplified coding sequence for residues 1–60 of c-Ha-Ras and residues 61–184 (including the termination codon) of rap1B. QuickChange mutagenesis (Stratagene, La Jolla, CA, U.S.A.) was used to change amino acids 179 and 180 from serines to alanines in ras/rap1B. All enhanced yellow fluorescent protein (EYFP) (Ormo *et al*., 1996)-tagged receptors were constructed using PCR to remove the receptor termination codons, and to introduce *Sac*I restriction sites immediately downstream of the final amino-acid codon in frame with the coding sequence of EYFP. The resulting PCR product was digested with *Sac*I and *Xho*I and ligated with EYFP. The adenylyl cyclase type II (AC2) clone used in these studies has been described ((Ma *et al*., 2004); generous gift of Dr P. Ram). The Gαo clone used in these studies has been described previously (Jones & Reed, 1987). All clones were subcloned into the pSI vector (Promega, Madison, WI, U.S.A.) and sequence verified before use.

Functional assays

Receptor Selection and Amplification Technology (R-SAT™) assays were performed as described (Ma *et al*., 2004) with the following modifications. Briefly, cells were plated one day before transfection using 7×10^3 cells in 0.1 ml of media per well of a 96-well plate. Cells were transiently transfected with 5 ng of receptor DNA, 20 ng ras/rap1B (AA), 2 ng AC2, and 30 ng pSI-β-galactosidase (Promega, Madison, WI, U.S.A.) per well of a 96-well plate using Polyfect (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. The use of ras/rap1B (AA) and AC2 was found to improve responses of GPCRs in this functional assay (Ma *et al*., 2004). At 1 day after transfection, the medium was changed and cells were combined with ligands in DMEM supplemented with 25% ultraculture synthetic supplement (Cambrex, Walkersville, MD, U.S.A.) 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 (Ma *et al*., 2004). Cells were rinsed with phosphate-buffered saline (PBS), pH = 7.4, before the addition of 200 μl PBS supplemented with 3.5 mM *O*-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, St Louis, MO, U.S.A.). The cells were incubated for 3 h before the plates were read at 420 nm on a plate reader (Bio-Tek EL 310 or Molecular Devices).

Ca^{2+} mobilization assays

Intracellular changes in calcium concentrations due to activation of Mrg's were detected using the calcium-binding bioluminescence protein aequorin, which was expressed as part of a tripartite chimeric protein: MT-GFP-AEQ, where the MT protein moiety (leader sequence of cytochrome *c* oxidase 8) targets the fusion protein to the inner membrane of the mitochondria (Baubet *et al*., 2000). HEK293T cells were transiently transfected with MrgX receptors and MT-GFP-AEQ in 10 cm cell culture dishes. At 2 days post-transfection; cells were washed once with PBS. The active MT-GFP-AEQ protein was obtained by overlaying cells in 10 cm dishes with 3 ml of loading solution (unsupplemented DMEM, 10 μM coelenteracine cp, 1 mM reduced glutathione, and 0.1% FBS). Cells were incubated for at least 4 h at 37°C protected from light. One wash with PBS-EDTA (Invitrogen-Gibco, Carlsbad, CA, U.S.A.) preceded harvesting the cells in KRB-calcium buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5.5 mM glucose). Cells were washed two additional times in KRB-calcium and resuspended to 2×10^6 cells ml^{-1} in KRB-calcium buffer. Kinetic luminescence measurements were performed with an automated microplate luminescence reader (Lumistar Galaxy, BMG Labtechnologies, Durham, NC, U.S.A.). Ligand dilutions were prepared in Costar 3903 white 96-well flat clear bottom microplates (Corning, Acton, MA, U.S.A.). A volume of 100 μl of transfected cells was automatically injected to initiate calcium responses and start 30 s measurements of total luminescence emission per well. The plates were shaken at 200 rounds min^{-1} for 1 s after injection. Also, 100 μl 1% Triton-X-100 in KRB-calcium was automatically added to each well after the first ligand-induced recordings to obtain total unused aequorin luminescence. Each measurement was expressed as percentage of total luminescence (used + unused). All luminescence traces were analyzed with the LUMIstar control software and Excel.

Membrane preparations

A total of 12 million HEK293T cells were plated in 15 cm dishes and transfected with 12.5 μg each of receptor and Gαo plasmid DNA using Polyfect (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested with 10 mM EDTA, in magnesium- and calcium-free Dulbecco's phosphate-buffered saline, resuspended in membrane buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, pH 7.2), and homogenized by 30 strokes with a tight-fitting plunger in a Dounce Homogenizer. The homogenate was spun at $1000 \times g$ for 10 min at 4°C to remove the nuclei and cellular debris. The supernatant was taken and spun at $45{,}000 \times g$ for 45 min at 4°C to collect the membrane fraction. Membrane protein concentrations were determined using a BioRad (Hercules, CA, U.S.A.) protein assay kit as per the manufacturer's instructions. Membranes were resuspended in membrane buffer to a final concentration of 1 mg ml^{-1}, snap frozen as aliquots in liquid nitrogen, and stored at −80°C.

GTP-γS-binding assays

In all, 5 μg of membranes was incubated at 37°C for 30 min in assay buffer (50 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, pH 7.2) in the presence of 10 μM GDP, 0.5 nM ^{35}S-GTP-γS (Perkin-Elmer Life Sciences, Shelton, CT, U.S.A.) and varying concentrations of agonist (total volume 100 μl in a 96-well plate). Membranes were filtered onto a 96-well GF/B filter plate (Packard Bioscience, Shelton, CT, U.S.A.) and washed with 500 ml wash buffer (50 mM Tris-HCl, 5 mM $MgCl_2$) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL, U.S.A.). The filter plates were dried under a heat lamp before addition of 50 μl of scintillation fluid to each well (Microscint 20, Packard, Shelton, CT, U.S.A.) and counted on a Topcount NXT (Packard, Shelton, CT, U.S.A.).

Measurement of expression levels

EYFP-tagged receptors were transfected into NIH-3T3 cells (40 ng per well of a 96-well plate) using Polyfect (Qiagen Valencia, CA, U.S.A.) as described above. At 1 day after transfection the medium was changed, and 3 days after transfection the medium was removed and fluorescence was quantified using a Mithras LB 940 Multilable Reader (excitation filter: 485 nm/emission filter: 535 nm; Berthold Technologies).

Data analysis

Concentration–response graphs for all functional assays were plotted and EC_{50} values were determined by nonlinear regression analysis using Prism software (GraphPad version 4.0, San Diego, CA, U.S.A.) according to the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\hat{}((\log EC_{50} - X)))$$

where X is the logarithm of concentration, Y is the response; Y starts at the bottom and goes to the top with a sigmoid shape. Allowing the Hill coefficient to vary did not significantly change the fits of the curves except for a few curves where no plateau was reached, where allowing the Hill coefficient to vary did not allow a reasonable fit of the data. Thus, the Hill coefficient was constrained to unity.

Results

Rhesus orthologs to MrgX1, MrgX2, MrgX3, and MrgX4 were cloned by PCR-based strategies and aligned with their human orthologs (Figure 1a–d). Also included in the alignment were human clones MrgX6 (also SNSR2, Genbank #AF474988) and MrgX7 (also Drr3 or SNSR3, Genbank #AF474989). A novel variant of human MrgX1 (hMrgX1-2) was identified, which differed by four amino-acid residues located in the carboxy-terminus of the receptor. Two variants of rhesus MrgX2 were identified (Figure 1b), and confirmed to exist in multiple individuals (not shown). Besides containing several amino-acid changes, rhesus MrgX2 variant 2 (rhMrgX2-2) contained one less amino-acid residue in transmembrane domain 3 (TMD3) than variant 1. Also, a variant of human Drr5/SNSR5 was identified that differed from the previously described clone (Genbank #AF474991) by one residue (L8F). Interestingly, the corresponding rhesus receptor is also L at position 8. Based on its high degree of similarity to human MrgX4, we have renamed this receptor human MrgX4, variant 2 (hMrgX4-2, Figure 1e).

The rhesus orthologs exhibited on average 87–93% sequence identity at the amino-acid level to their human counterparts, with the exception of the rhesus MrgX4 receptor, which had only 80% identity with its human counterparts (Figure 1). In contrast, none of the rodent receptors identified to date have more than 50% identity to the human receptors (Dong *et al*., 2001). The amino-acid differences between rhesus and human MrgX receptors were distributed throughout the receptors, including domains predicted to form extracellular loops, intracellular loops, and transmembrane domain helices, though the differences tended to occur more frequently in the N- and C-terminal regions. The relatedness of these receptors is shown in Figure 1e.

As many pseudogenes are found within the Mrg family (Dong *et al*., 2001), it was important to demonstrate the functionality of these novel receptors. To characterize their signal transduction properties, we employed a cell-based proliferation assay that is compatible with all classes of GPCRs, which is based on the observation that oncogenes and many receptors induce proliferation or transformation responses in NIH-3T3 cells. These growth-stimulatory effects can be reliably detected and quantified using a cotransfected

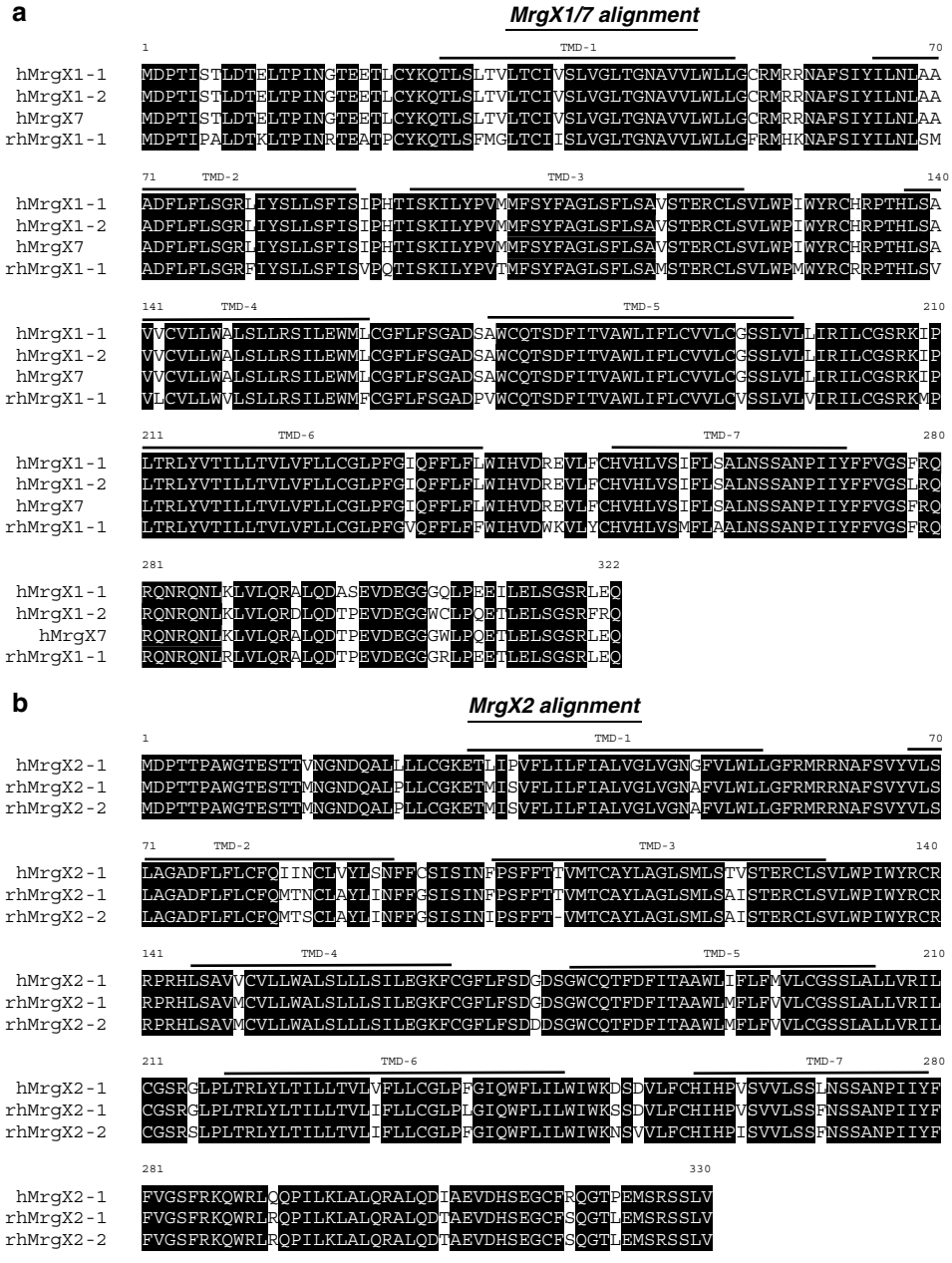

Figure 1 Alignment of rhesus and human MrgX1/X7 (a), MrgX2 (b), MrgX3/X6 (c), and MrgX4/X5 (d). Receptors were cloned by PCR as described in Methods. The human genes have been described (Dong *et al*., 2001; Lembo *et al*., 2002). The rhesus gene sequences have been deposited in Genbank with the following accession numbers (pending). Dendogram of MrgX receptors (e); a dendogram of the MrgX receptors cloned from humans and rhesus monkeys was constructed using DS Gene (Version 1.5, Accelrys, San Diego, CA, U.S.A.) UPGMA analysis. Numbers represent the average change per residue.

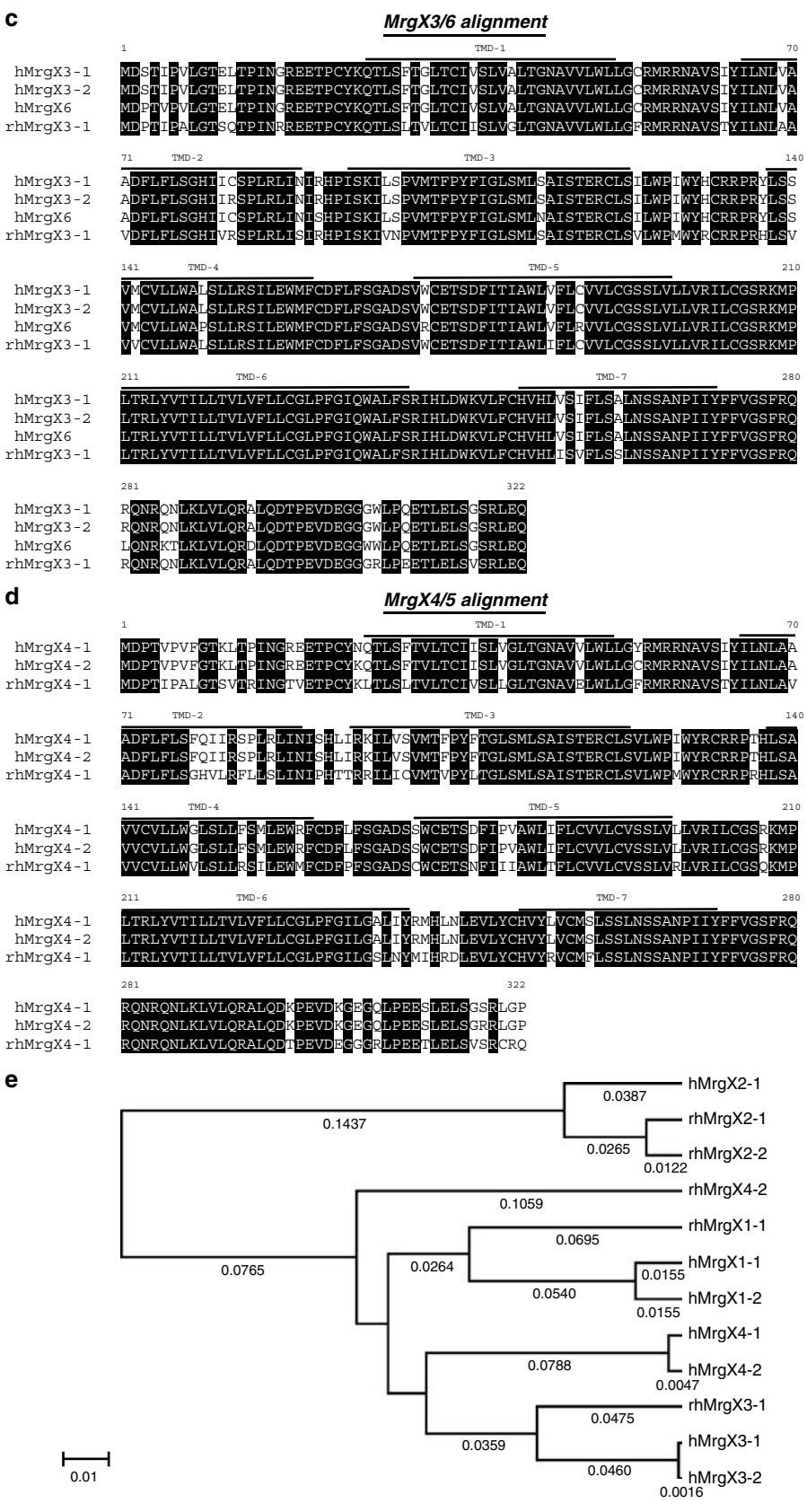

Figure 1 Continued

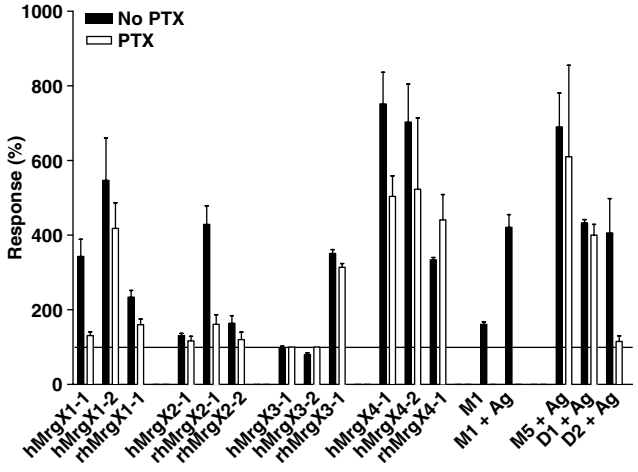

Figure 2 Constitutive activity – cellular proliferation. The indicated receptors were each transfected (6 ng per well of a 96-well dish) into NIH-3T3 cells and analyzed for constitutive proliferative responses as described in Methods. Receptors were cotransfected with a ras/rap chimera (20 ng per well) and adenylate cyclase type II (AC2, 2 ng per well) to allow simultaneous detection of Gq-, Gi-, and Gs-mediated responses, as described previously (Weissman *et al*., 2004). Where indicated, PTX was added (100 ng ml^{-1}) 24 h post-transfection. Control transfections contained empty vector, ras/rap and AC2. Responses for control receptors were measured in the presence of agonist where indicated (Ag = 20 μM carbachol for the M1 and M5 muscarinic receptors, 10 μM SKF 38393 for D1 dopamine receptor and 1 μM pergolide for D2 dopamine receptor). Responses were normalized to the basal response of the control transfections, which were assigned a value of 100% (denoted by the horizontal line). Responses are the average ± s.e.m. of at least three independent experiments comprised of at least four determinations per experiment.

marker gene (β-galactosidase; see Ma *et al*., 2004; Weissman *et al*., 2004).

The human and rhesus MrgX receptors were transiently expressed, and analyzed for their abilities to constitutively activate cellular proliferation. Human MrgX1 and MrgX4, as well as rhesus MrgX2, MrgX3, and MrgX4, all produced robust constitutive responses (Figure 2, Table 1), comparable to or greater agonist-stimulated responses of prototypical Gq-, Gi-, and Gs-coupled GPCRs, the M1 and M5 muscarinic receptors, the D2 dopamine receptor, and the D1 dopamine receptor, respectively. The rhMrgX1-1 and rhMrgX2-2 receptors produced small but reproducible constitutive responses, while the hMrgX2-1, hMrgX3-1, and hMrgX3-2 receptors produced little or no constitutive response.

To distinguish whether the observed constitutive activity was through Gi-regulated pathways, receptors were assayed in the presence of pertussis toxin (PTX). PTX pretreatment significantly reduced the constitutive response to hMrgX1-1 and rhMrgX2-1, indicating, these receptors may utilize PTX-sensitive G-proteins to signal. In contrast, PTX did not significantly reduce the constitutive activity observed for hMrgX1-2, or rhMrgX3-1, rhMrgX4-1, hMrgX4-1, or hMrgX4-2. The robust constitutive activity of the MrgX4 receptors observed in the cellular proliferation assay was also seen in phosphatidyl inositol (PI) hydrolysis assays, confirming that the constitutive responses were Gq mediated (data not shown).

To determine whether or not the observed differences in constitutive activity were due to differences in expression levels, all the MrgX receptors were tagged with the fluorescent

Table 1 Constitutive activity – cellular proliferation

Receptor	*Species*	*Variant*	*Response* (%)	*PTX Response* (%)	*Expression* (%)
MrgX1	Human	1	343 ± 46	131 ± 5	174 ± 14
	Human	2	558 ± 110	417 ± 35	143 ± 11
	Rhesus	1	234 ± 18	160 ± 8	80 ± 7
MrgX2	Human	1	131 ± 6	117 ± 6	85 ± 10
	Rhesus	1	429 ± 49	161 ± 13	57 ± 10
	Rhesus	2	164 ± 20	120 ± 8	44 ± 9
MrgX3	Human	1	96 ± 7	ND	26 ± 12
	Human	2	80 ± 5	ND	33 ± 11
	Rhesus	1	351 ± 10	314 ± 7	33 ± 11
MrgX4	Human	1	752 ± 84	504 ± 22	46 ± 12
	Human	2	635 ± 102	431 ± 84	40 ± 9
	Rhesus	1	334 ± 7	441 ± 48	66 ± 12
M1	Human		161 ± 11	ND	100 ± 11
M1 + Ag	Human		421 ± 59	ND	100 ± 41
M5 + Ag	Human		615 ± 93	547 ± 86	ND
D1 + Ag	Human		335 ± 57	358 ± 30	ND
D2 + Ag	Human		364 ± 62	113 ± 5	ND

The indicated receptors were each transfected with a ras/rap chimera and adenylate cyclase type II (AC2) into NIH-3T3 cells and analyzed for constitutive proliferative responses as described in Methods. The use of ras/rap and AC2 allows simultaneous detection of Gq-, Gi-, and Gs-mediated responses (Weissman *et al*., 2004). The D1 dopamine receptor, the D2 dopamine receptor, and the M_5 muscarinic receptor were stimulated with 10 μM SKF38393, 1 μM pergolide, and 20 μM carbachol, respectively. Where indicated, PTX was added (100 ng ml^{-1}) 24 h post-transfection. Control transfections contained empty vector, ras/rap, and AC2. Responses were normalized to the basal response of the control transfections, which were assigned a value of 100%. Responses are the average ± s.e.m. of at least three independent experiments comprised of at least four determinations per experiment. Receptor expression levels were assessed by transfecting YFP-tagged receptors into NIH-3T3 cells and measuring luminescence as described in Methods. The fluorescence values for each receptor were normalized to the value for the M_1 muscarinic receptor, which was 8103 relative fluorescence units. Numbers represent the average ± s.e.m. of 10 determinations. ND = not determined.

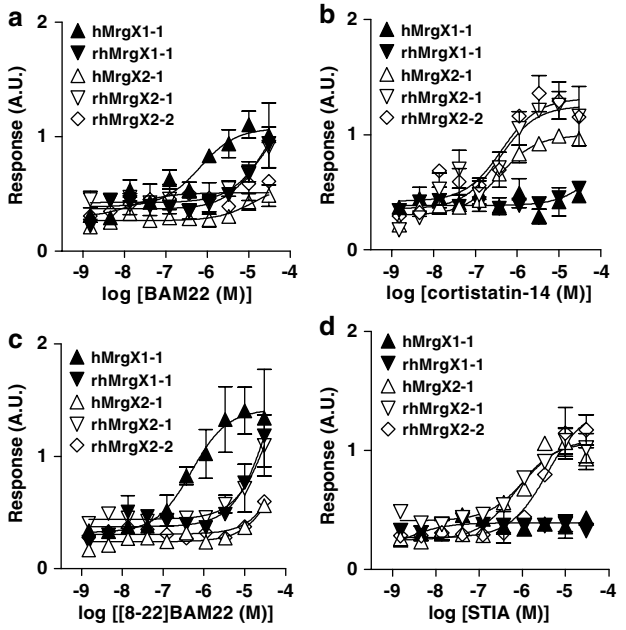

Figure 3 MrgX1–X2 pharmacology – cellular proliferation. The indicated receptors were each transfected (6 ng per well of a 96-well dish) with ras/rap (20 ng per well) and AC2 (2 ng per well) into NIH-3T3 cells and analyzed for proliferative responses in the presence of the indicated concentrations of (a) BAM22, (b) STIA, (c) [8–22]BAM22, and (d) cortistatin-14, as described in Methods. Data points are the means of two determinations ± s.d.

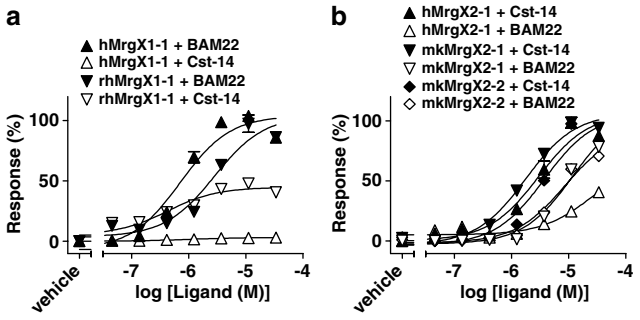

Figure 4 MrgX1–X2 pharmacology – Ca^{2+} mobilization. The human and rhesus MrgX1 and MrgX2 receptors were each transfected (10 μg per 15 cm^2 dish) with ceolenterazine (10 μg per 15 cm^3 dish) into HEK293T cells. Cells were harvested and analyzed for Ca^{2+} release in the presence of the indicated ligands as described in Methods. Data points are the means of two determinations ± s.d. (a) Ligand responses of hMrgX1 and rhMrgX1 were normalized to their responses to BAM22, which were 41 and 4 relative luminescence units (RLUs), respectively. (b) Ligand responses of hMrgX2, rhMrgX2-1, and rhMrgX2-2 were normalized to their responses to cortistatin-14, which were 8, 35, and 14 RLUs, respectively.

protein EYFP and expression directly analyzed by measuring fluorescence. As shown in Table 1, the expression levels of these receptors varied considerably; however, there was no correlation between high levels of expression and high constitutive activity. In fact, several of the most constitutively active receptors (e.g. the MrgX4 receptors) were among the most poorly expressed. In comparison to the M_1 muscarinic receptor, which has minimal constitutive activity (see Table 1 and Figure 2, M_1 with no agonist), many of the MrgX receptors were less well expressed, although many had high levels of constitutive activity. Separate experiments verified that the constitutive activity of these receptors was not significantly altered by the introduction of the fluorescent tags, nor was the M_1 response to carbachol (data not shown).

BAM22 and related peptides, as well as cortistatin-14 and STIA, have been described as agonist ligands to the human MrgX1 and human MrgX2 receptors, respectively (Lembo *et al*., 2002; Robas *et al*., 2003) (patent #WO 01/98330 A2). We therefore tested these peptides at the human and rhesus variants of MrgX1 and MrgX2 described above in a variety of functional assays. In the cellular proliferation assay, cortistatin-14 and STIA activated both human and rhesus MrgX2 receptors with similar potencies, but displayed little or no activity at either the human or rhesus MrgX1 receptors (Figure 3, Table 2). Potencies ranged from 0.5 to 2 μM for cortistatin-14, and between 2 and 4 μM for STIA at the MrgX2 receptors. In contrast, BAM22 and the related peptide [8–22]BAM22 were significantly less potent at rhMrgX1-1 compared to hMrgX1-1 receptor, and were also able to activate both rhesus MrgX2 receptors.

To validate results obtained using the cellular proliferation assay, and to better define the signaling properties of the rhesus MrgX1 and MrgX2 receptors, these peptides were profiled in Ca^{2+}-releasing assays, and GTP-γS-binding assays. In Ca^{2+}-releasing assays, cortistatin-14 displayed selectivity for both rhesus and human MrgX2 receptors over MrgX1 receptors, although a small degree of activation of rhMrgX1-1 was observed (Figure 4, Table 2). Potencies observed ranged between 1 and 3 μM, similar to results using cellular proliferation. Also in agreement with the cellular proliferation results, BAM22 and [8–22]BAM22 were significantly less potent at rhMrgX1-1 than at hMrgX1-1, though they did display greater selectivity between rhesus MrgX1 and rhesus MrgX2 receptors than in the cellular proliferation assay.

As a significant proportion of the constitutive proliferative responses induced by MrgX1 and MrgX2 receptors were blocked by PTX, the MrgX1 and MrgX2 receptors were tested for the ability to activate GTP-γS binding to Gαo, a PTX-sensitive G-protein highly expressed in neuronal tissues (Brann *et al*., 1987). Both human and rhesus MrgX1 and MrgX2 receptors were able to stimulate GTP-γS binding when challenged with BAM22 and cortistatin-14, respectively (Figure 5, Table 2). As observed in the other functional assays, cortistatin-14 exhibited strong selectivity for both rhesus and human MrgX2 receptors, whereas BAM22 and the related peptide [8–22]BAM22 displayed a preference for human MrgX1 over rhesus MrgX1 receptors, and were able to modestly activate the rhesus MrgX2 receptor variants.

Conclusions

We have cloned a novel family of rhesus Mrg receptors. Structurally, the rhesus receptors represent true orthologs to the human MrgX receptors, as well as the recently described MrgX receptors in macaque (Zhang *et al*., 2005). Several novel variants of these receptors were described, including one rhesus MrgX2 variant, one human MrgX1 variant, and one human MrgX4 variant. RhMrgX2-2 was unique in that it encoded a receptor containing one less amino-acid in TMD3 than either the hMrgX2-1 or rhMrgX2-1. We identified this

Table 2 MrgX1–X2 pharmacology

			Cortistatin-14		*STIA*		*BAM22*		*[8–22]BAM22*	
Receptor	*Species*	*Variant*	pEC_{50}	*Resp* (%)	pEC_{50}	*Resp* (%)	pEC_{50}	*Resp* (%)	pEC_{50}	*Resp* (%)
Cellular proliferation										
MrgX1	Human	1	—	15±9	—	15±10	6.1±0.6	100±14	5.9±0.6	100±26
	Rhesus	1	—	9±2	—	1–	4.5±0.6	50±25	4.2±0.2	63±11
MrgX2	Human	1	6.3±0.6	100±28	5.6±0.4	100±15	<4.5–	17±13	<4.5–	34±7
	Rhesus	1	6.1±0.4	86±25	5.5±0.5	121±44	5±0.5	27±35	<4.5–	39±47
	Rhesus	2	5.8±0.5	101±0	5.6±0.2	111±21	<4.5–	32±6	<4.5–	32±6
Ca^{2+} mobilization										
MrgX1	Human	1	—	12±11	—	5±2	6.6±0.4	100±0	7.6±0.0	100–
	Rhesus	1	6.5±0.4	29±13	—	3±1	5.6±0.4	100±0	6.2±0.0	100–
MrgX2	Human	1	5.8±0.3	100–	5.7±0.2	93±12	4.8±0.2	50±11	4.4±0.2	37±15
	Rhesus	1	6.0±0.3	100–	5.7±0.1	102±4	5.1±0.3	92±15	4.4±0.1	72±23
	Rhesus	2	5.7±0.4	100–	5.1±0.1	106±10	5.2±0.2	89±3	4.4±0.1	75±48
GTPγS-binding										
MrgX1	Human	1	—	0–	—	0–	6.4±0.6	162±30	6.5±0.3	180±23
	Rhesus	1	—	4±7	—	7±12	5.1±0.7	33±25	4.5±0.6	66±65
MrgX2	Human	1	5.9±0.3	62±18	4.5±0.3	94±53	4.5±0.2	54±23	—	4±8
	Rhesus	1	6.0±0.3	61±27	4.9±0.3	56±11	4.8±0.4	64±32	4.8±0.4	15±4
	Rhesus	2	4.8±0.5	68±31	<4.5–	33±17	5±0.5	41±30	—	2±5

Cellular proliferation: The indicated receptors were each transfected (6 ng per well of a 96-well dish) with ras/rap (20 ng per well) and AC2 (2 ng per well) into NIH-3T3 cells and analyzed for proliferative responses in the presence of the indicated concentrations of peptides as described in Methods. Results are the average±s.d. of at least two independent experiments each carried out with duplicate determinations for each data point. Responses of all receptors to cortistatin-14 and STIA were normalized to the response of hMrgX2, which were approximately 1.2 absorbance units in each case. Responses of all receptors to BAM22 and [8–22]BAM22 were normalized to the response of hMrgX1, which were approximately 1 and 1.3 absorbance units each, respectively.
Ca^{2+} mobilization: The indicated receptors were each transfected (10 μg per 15 cm^2 dish) with ceolenterazine (10 μg per 15 cm^3 dish) into HEK293 cells, cells harvested, and analyzed for Ca^{2+} release in the presence of the indicated concentrations of peptides as described in Methods. Results are the average±s.d. of at least two independent experiments each carried out with duplicate determinations for each data point. Ligand responses of hMrgX1 and rhMrgX1 were normalized to their responses to BAM22, which were 41 and 4 relative luminescence units (RLUs), respectively. Ligand responses of hMrgX2, rhMrgX2-1, and rhMrgX2-2 were normalized to their responses to Cortistatin-14, which were 8, 35, and 14 RLUs, respectively.
GTP-γS binding: The indicated receptors were each transfected (12.5 μg per 15 cm^2 dish) into HEK293 cells, membranes prepared, and analyzed for GTP-γS binding in the presence of the indicated concentrations of peptides as described in Methods. Data are presented as the average percent GTP-γS binding above basal level±s.d. of at least two independent experiments each carried out with duplicate determinations for each data point. For all assays, where indicated, a dash indicates a pEC50 could not be determined, a 'less than' symbol (<) indicates that the pEC50 is estimated to be less than the given number, and that the response shown was determined at the highest concentration of ligand tested.

variant in six of eight individuals, and demonstrate that, despite this radical change, it is a functional receptor with properties similar to rhMrgX2-1, and to hMrgX2-1. Sequence fragments containing this gap have been described (e.g. ti #349921200, 561736400, 455527434, and 496076704 in the NCBI *M. mulatta* trace archive), while partial sequences containing the longer variant have also been reported (e.g. ti #501549690, 503069495 and 542050281 in the NCBI *M. mulatta* trace archive). The high degree of polymorphism we observed for the MrgX receptors is consistent with the findings that polymorphisms are more prevalent in genes with restricted expression patterns (Duret & Mouchiroud, 2000).

Functional characterization demonstrated that the majority of these receptors are constitutively active, with many displaying high constitutive activity. Estimation of expression levels using fluorescent tags revealed that the high constitutive activity of these receptors was not due to high expression of the receptors. Further analysis revealed that both the human and the rhesus MrgX1 and MrgX2 receptors couple to Gq- and Gi-regulated pathways, whereas the human and rhesus MrgX3 and MrgX4 receptors couple primarily to Gq-regulated pathways. Coupling to Gq-regulated pathways was confirmed using Ca^{2+} release assays and coupling to Gi-regulated pathways was demonstrated with PTX, and confirmed with GTP-γS-binding assays. Overall, there was a high degree of functional conservation between homologous pairs of rhesus and human receptors with respect to both the level of constitutive activity, and G-protein-coupling preference.

Where comparisons are possible, our results agree well with previous studies on the functional properties of the Mrg receptors. MrgX1 (Lembo *et al*., 2002), MrgX2 (Robas *et al*., 2003), and the mouse receptors MrgA1 and MrgC11 are known to couple Gq (Han *et al*., 2002). However, none of these studies noted constitutive activity of these receptors. More recently, it was shown that MrgX1 heterologously expressed in cultured rat neurons couples to Gq proteins and PTX-sensitive Gi/o proteins, the latter constitutively (Chen & Ikeda, 2004). This finding agrees with our observations for human MrgX1.

Given that many of the MrgX receptors may be constitutively active *in vivo*, it is tempting to speculate on the possible physiological roles constitutive activity may play. Assuming a role in sensory detection, constitutive activity may reflect a partially 'on state' of the receptors so that they are continually monitoring both increases and decreases in sensory inputs. Thus, the receptors that are more highly constitutively active may provide basal sensory input of environmental conditions, whereas the less active receptors become turned on only when

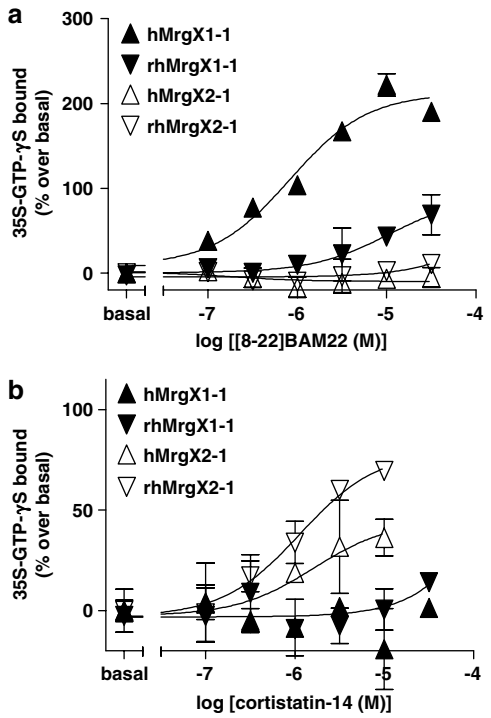

Figure 5 MrgX1–X2 pharmacology – GTP-γS binding. The indicated receptors were each transfected with Gαo (12.5 μg each of receptor and G-protein per 15 cm^3 dish) into HEK293T cells. Membranes were prepared as described in Methods and analyzed for GTP-γS binding in the presence of the indicated concentrations of (a) [8–22]BAM22 or (b) cortistatin-14, as described in Methods. Basal level of GTP-γS binding was measured in the absence of ligand. Data are presented as the average percent GTP-γS binding above basal $\pm$ s.d. of duplicate points.

drastic changes (e.g., acute pain associated with physical trauma) in sensory inputs occur. The high degree of constitutive activity implies that endogenous inverse agonists for the MrgX receptors may exist, although none have been identified to date. Such a precedent exists with the endogenous peptide agouti, which acts as an endogenous inverse agonist at the melanocortin 4 receptor (Nijenhuis *et al*., 2001).

Diverse ligands have been identified for several members of the Mrg family, including RF-amide peptides (Dong *et al*., 2001), BAM22-related peptides (Lembo *et al*., 2002), β-alanine (Shinohara *et al*., 2004), angiotensin (1–7) (Santos *et al*., 2003), and adenine (Bender *et al*., 2002). Whether these represent true endogenous regulators of the Mrg receptors is unclear. Several of these ligands are implicated in nociception, such as the RF-amide neuropeptides and the series of proenkephalin A-related peptides, exemplified by BAM22 (2, 4). Cortistatin-14 was proposed to be the authentic ligand for MrgX2 based on the fact that it was the most potent ligand identified in a functional screen and that it is coexpressed with MrgX2 in the DRG (Robas *et al*., 2003). Cortistatin-14 is known to mediate several physiological effects unrelated to nociception, including induction of slow wave sleep (Spier & de Lecea, 2000). Given that cortistatin-14 is expressed in many tissues where MrgX2 is not found, it probably mediates some of its effects through other receptors.

We have observed that both BAM-22 and cortistatin-14 activate the corresponding rhesus orthologs. In general, cortistatin-14 displayed strong selectivity for both human and rhesus MrgX2 receptors over MrgX1 receptors in all functional assays used, and similar activity at rhesus and human MrgX2 receptors. In contrast, BAM22 and [8–22]BAM22 displayed significantly lower activity at rhesus MrgX1 than at human MrgX1, and they were not as selective between rhesus MrgX1 and rhesus MrgX2 receptors as were cortistatin-14 and STIA. Thus, whether or not BAM22 represents the true endogenous activator of human MrgX1, it seems unlikely to fulfill this role for rhesus MrgX1. While this manuscript was in preparation, the proadrenomedullin *N*-terminal peptides PAMP-20 and PAMP-12 were described as ligands for MrgX2 (Kamohara *et al*., 2005). We confirmed that these peptides activate hMrgX2-1 and found that they have comparable activity on rhMrgX2-1 and rhMrgX2-2 (unpublished observations). Overall these results indicate significant pharmacological conservation between human and rhesus MrgX receptors.

One of the most impressive features of the Mrg receptor family is the extraordinarily discrete localization of many subtypes to small-diameter sensory neurons in DRG. In mouse, the MrgA subgroup and MrgD are expressed in nonoverlapping populations of sensory neurons, implying that the individual subtypes may play distinct roles in sensation (Dong *et al*., 2001). Interestingly, of the mouse Mrg receptors, the MrgA subgroup is the most closely related by phylogenetic analysis to the human MrgX receptors. Very limited localization studies have been performed on the human MrgX receptors, although they are also expressed in DRG. Recently it was shown that, like rodent and human genes, the macaque Mrg receptors are also expressed in DRG neurons, and that the MrgX2 subtype colocalizes with known nociceptive markers IB4, VR1, and SP (Zhang *et al*., 2005). From these studies, it seems likely that mouse MrgA receptors perform similar functions as the human and rhesus MrgX receptors.

Several approaches should eventually clarify the functions of the MrgX receptors. One is the reverse pharmacologic method described recently (Dong *et al*., 2001; Lembo *et al*., 2002; Robas *et al*., 2003). However, this approach suffers from the fact that the complete pharmacological profiles of the identified ligands are poorly defined. Another is a genetic approach, which was first used to isolate sensory neuron-specific Mrg receptors (Dong *et al*., 2001). That study exploited the fact that mice lacking the transcription factor neurogenin 1 do not develop sensory neurons to identify SNSRs. However, knockouts of the murine Mrg subtypes are unlikely to define specific roles for the human MrgX receptors, given that orthologs do not exist.

An approach that may ultimately prove most productive for elucidating the physiological roles of the MrgX receptors is chemical genomics (Lenz *et al*., 2000; Caron *et al*., 2001). This method entails first discovering and developing potent, selective small-molecule surrogate agonist and antagonist ligands for each of the receptor subtypes, and then testing them in appropriate *in vivo* models. Utilizing the information described above on the functional properties of the MrgX receptors, high-throughput screening efforts have been initiated to identify small-molecule modulators of the MrgX receptors. It is expected that *in vivo* testing of such compounds, should they become available, will help elucidate the functions of these genes.

We thank M. Brunette and E. Pham for excellent technical assistance.

References

BAUBET, V., LE MOUELLIC, H., CAMPBELL, A.K., LUCAS-MEUNIER, E., FOSSIER, P. & BRULET, P. (2000). Chimeric green fluorescent protein-aequorin as bioluminescent Ca^{2+} reporters at the single-cell level. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 7260–7265.

BENDER, E., BUIST, A., JURZAK, M., LANGLOIS, X., BAGGERMAN, G., VERHASSELT, P., ERCKEN, M., GUO, H.Q., WINTMOLDERS, C., VAN DEN WYNGAERT, I., VAN OERS, I., SCHOOFS, L. & LUYTEN, W. (2002). Characterization of an orphan G protein-coupled receptor localized in the dorsal root ganglia reveals adenine as a signaling molecule. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 8573–8578.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science,* **237,** 527–532.

BONNER, T.I., YOUNG, A.C., BRANN, M.R. & BUCKLEY, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron,* **1,** 403–410.

BRANN, M.R., COLLINS, R.M. & SPIEGEL, A. (1987). Localization of mRNAs encoding the alpha-subunits of signal-transducing G-proteins within rat brain and among peripheral tissues. *FEBS Lett.,* **222,** 191–198.

CARON, P.R., MULLICAN, M.D., MASHAL, R.D., WILSON, K.P., SU, M.S. & MURCKO, M.A. (2001). Chemogenomic approaches to drug discovery. *Curr. Opin. Chem. Biol.,* **5,** 464–470.

CHEN, H. & IKEDA, S.R. (2004). Modulation of ion channels and synaptic transmission by a human sensory neuron-specific G-protein-coupled receptor, SNSR4/mrgX1, heterologously expressed in cultured rat neurons. *J. Neurosci.,* **24,** 5044–5053.

DONG, X., HAN, S., ZYLKA, M.J., SIMON, M.I. & ANDERSON, D.J. (2001). A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell,* **106,** 619–632.

DURET, L. & MOUCHIROUD, D. (2000). Determinants of substitution rates in mammalian genes: expression pattern affects selection intensity but not mutation rate. *Mol. Biol. Evol.,* **17,** 68–74.

HAN, S.K., DONG, X., HWANG, J.I., ZYLKA, M.J., ANDERSON, D.J. & SIMON, M.I. (2002). Orphan G protein-coupled receptors MrgA1 and MrgC11 are distinctively activated by RF-amide-related peptides through the Galpha q/11 pathway. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 14740–14745.

JONES, D.T. & REED, R.R. (1987). Molecular cloning of five GTP-binding protein cDNA species from rat olfactory epithelium. *J. Biol. Chem.,* **262,** 14241–14249.

JULIUS, D. & BASBAUM, A.I. (2001). Molecular mechanisms of nociception. *Nature,* **413,** 203–210.

KAMOHARA, M., MATSUO, A., TAKASAKI, J., KOHDA, M., MATSUMOTO, M., MATSUMOTO, S., SOGA, T., HIYAMA, H., KOBORI, M. & KATOU, M. (2005). Identification of MrgX2 as a human G-protein-coupled receptor for proadrenomedullin *N*-terminal peptides. *Biochem. Biophys. Res. Commun.,* **330,** 1146–1152.

LEMBO, P.M., GRAZZINI, E., GROBLEWSKI, T., O'DONNELL, D., ROY, M.O., ZHANG, J., HOFFERT, C., CAO, J., SCHMIDT, R., PELLETIER, M., LABARRE, M., GOSSELIN, M., FORTIN, Y., BANVILLE, D., SHEN, S.H., STROM, P., PAYZA, K., DRAY, A., WALKER, P. & AHMAD, S. (2002). Proenkephalin A gene products activate a new family of sensory neuron-specific GPCRs. *Nat. Neurosci.,* **5,** 201–209.

LENZ, G.R., NASH, H.M. & JINDAL, S. (2000). Chemical ligands, genomics and drug discovery. *Drug Discov. Today,* **5,** 145–156.

MA, J.N., CURRIER, E.A., ESSEX, A., FEDDOCK, M., SPALDING, T.A., NASH, N.R., BRANN, M.R. & BURSTEIN, E.S. (2004). Discovery of novel peptide/receptor interactions: identification of PHM-27 as a potent agonist of the human calcitonin receptor. *Biochem. Pharmacol.,* **67,** 1279–1284.

MA, Q., FODE, C., GUILLEMOT, F. & ANDERSON, D.J. (1999). Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev.,* **13,** 1717–1728.

NIJENHUIS, W.A., OOSTEROM, J. & ADAN, R.A. (2001). AgRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Mol. Endocrinol.,* **15,** 164–171.

ORMO, M., CUBITT, A.B., KALLIO, K., GROSS, L.A., TSIEN, R.Y. & REMINGTON, S.J. (1996). Crystal structure of the Aequorea victoria green fluorescent protein. *Science,* **273,** 1392–1395.

ROBAS, N., MEAD, E. & FIDOCK, M. (2003). MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion. *J. Biol. Chem.,* **278,** 44400–44404.

SANTOS, R.A., SIMOES E SILVA, A.C., MARIC, C., SILVA, D.M., MACHADO, R.P., DE BUHR, I., HERINGER-WALTHER, S., PINHEIRO, S.V., LOPES, M.T., BADER, M., MENDES, E.P., LEMOS, V.S., CAMPAGNOLE-SANTOS, M.J., SCHULTHEISS, H.P., SPETH, R. & WALTHER, T. (2003). Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 8258–8263.

SHINOHARA, T., HARADA, M., OGI, K., MARUYAMA, M., FUJII, R., TANAKA, H., FUKUSUMI, S., KOMATSU, H., HOSOYA, M., NOGUCHI, Y., WATANABE, T., MORIYA, T., ITOH, Y. & HINUMA, S. (2004). Identification of a G protein-coupled receptor specifically responsive to beta-alanine. *J. Biol. Chem.,* **279,** 23559–23564.

SNIDER, W.D. & MCMAHON, S.B. (1998). Tackling pain at the source: new ideas about nociceptors. *Neuron,* **20,** 629–632.

SPIER, A.D. & DE LECEA, L. (2000). Cortistatin: a member of the somatostatin neuropeptide family with distinct physiological functions. *Brain Res. Rev.,* **33,** 228–241.

WEISSMAN, J.T., MA, J.N., ESSEX, A., GAO, Y. & BURSTEIN, E.S. (2004). G-protein-coupled receptor-mediated activation of rap GTPases: characterization of a novel Galphai regulated pathway. *Oncogene,* **23,** 241–249.

YOUNG, D., WAITCHES, G., BIRCHMEIER, C., FASANO, O. & WIGLER, M. (1986). Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. *Cell,* **45,** 711–719.

ZHANG, L., TAYLOR, N., XIE, Y., FORD, R., JOHNSON, J., PAULSEN, J.E. & BATES, B. (2005). Cloning and expression of MRG receptors in macaque, mouse, and human. *Brain Res. Mol. Brain Res.,* **133,** 187–197.

ZYLKA, M.J., DONG, X., SOUTHWELL, A.L. & ANDERSON, D.J. (2003). Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 10043–10048.

(Received June 7, 2005
Revised September 1, 2005
Accepted September 11, 2005
Published online 14 November 2005)