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Interactions between the Mas-Related Receptors MrgD and MrgE Alter Signalling and Trafficking of MrgD

Sandra Milasta, John Pediani, Shirley Appelbe, Steven Trim, Michael Wyatt, Peter Cox, Mark Fidock, and Graeme Milligan

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom (S.M., J.P., S.A., G.M.); and Pfizer Global Research and Development, Sandwich, Kent, United Kingdom (S.T., M.W., P.C., M.F.)

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ABSTRACT

When expressed via an inducible promoter in human embryonic kidney 293 cells, the rat Mas-related gene D (rMrgD) receptor responded to β-alanine but not L-alanine by elevating intracellular [Ca^{2+}], stimulating phosphorylation of the mitogen-activated protein kinases known as extracellular signal-regulated kinase (ERK) 1 and ERK2 and translocating from the plasma membrane to punctate intracellular vesicles. By contrast, the related rat Mas-related gene E (rMrgE) receptor did not respond to β-alanine. Coexpression of rMrgD with rMrgE, which occurs in peripheral nociceptive neurons, allowed coimmunoprecipitation of the two receptors and resulted in the detection of cell surface rMrgD-rMrgE heterodimers via time-resolved fluorescence resonance energy transfer. These interactions increased the potency of β-alanine to phosphorylate ERK1 and ERK2 as well as maintaining the capacity of β-alanine to elevate intracellular [Ca^{2+}], which was reduced in magnitude and slowed in response with increasing times of expression of rMrgD in isolation. Associated with these effects, the presence of rMrgE restricted β-alanine-induced internalization of rMrgD. This is the first report of heterodimeric interactions between members of the Mas-related gene (Mrg) receptor family and indicates that interactions between rMrgD and rMrgE modulate the function of rMrgD. Because the Mrg receptors are potential therapeutic targets in pain, these results suggest that efforts to understand the function and regulation of individual Mrg family receptors may require coexpression of relevant pairs.

G protein-coupled receptors (GPCRs) of the Mas-related gene (Mrg) family (Dong et al., 2001) are selectively expressed in subpopulations of sensory neurons involved in the perception of pain (Dong et al., 2001; Lembo et al., 2002). This has resulted in their being given the additional name sensory neuron-specific G protein-coupled receptors (Lembo et al., 2002) and in suggestions that they might represent attractive targets for therapeutic intervention in pain. In mouse, the family is large, consisting of more than 50 members (Dong et al., 2001; Zylka et al., 2003), but the complement of related receptors in man, macaque, and rat is significantly less extensive (Zhang et al., 2005) although true orthologs are difficult to identify. In rat, for example, only single members of each of the MrgA and MrgC subfamilies have been identified (Zylka et al., 2003; Zhang et al., 2005), although, as in mouse, a substantial number of both true MrgB receptor encoding- and pseudogenes have been identified (Zylka et al., 2003). A single gene seems to encode the MrgD receptor in all rodent and primate species and this GPCR, which has also been named TGR7 (Shinohara et al., 2004), is activated selectively by β-alanine (Shinohara et al., 2004). By contrast, many of the other Mrg receptors have been shown to respond to relatively high concentrations of a range of peptide ligands (Dong et al., 2001; Han et al., 2002; Lembo et al., 2002; Robas et al., 2003; Grazzini et al., 2004) or remain orphans.

It is now widely accepted that GPCRs can form dimers, and this may be integral to function (Angers et al., 2002; Milligan et al., 2003; Breitwieser, 2004; Milligan, 2004). There is also growing evidence that certain GPCRs can form heterodimers when they are coexpressed (George et al., 2002; Milligan, 2004) and that such heterodimers may have pharmacology (Rocheville et al., 2000), function (Jordan and Devi, 1999), and regulation (Hansen and Sheikh, 2004) distinct from that of the corresponding homodimers. This has not been exam-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; Mrg, Mas-related gene; r, rat; Tr-FRET, time-resolved fluorescence resonance energy transfer; eYFP, enhanced yellow fluorescent protein; PCR, polymerase chain reaction; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; APC, allophycocyanin.

ined for members of the Mrg family; hence, as an initial effort to explore this issue, we selected the rat (r) MrgD and MrgE receptors because they are closely related and, as shown previously, are coexpressed in individual dorsal root ganglion neurons from a number of species (Zhang et al., 2005). However, whereas β-alanine acts as an agonist for MrgD, selective agonist ligands for MrgE have not yet been identified. Using cell lines able to express either rMrgD or rMrgE in an inducible manner or to induce expression of rMrgD in the face of constitutive expression of rMrgE, we confirm the agonist actions of β-alanine only on rMrgD and demonstrate that rMrgD and rMrgE interact directly upon coexpression and that interaction with rMrgE alters both the ability of β-alanine to internalize rMrgD and the potency of β-alanine to induce intracellular signals. These results demonstrate that Mrg receptors can form heterodimers and, by so doing, may alter their functionality and response to appropriate stimuli in sensory neurons.

Materials and Methods

Materials/Ligands

β-Alanine, L-alanine, doxycycline, and biotin-conjugated anti-c-myc antibody were supplied by Sigma (Gillingham, Dorset, UK), and all materials for tissue culture were from Invitrogen (Paisley, UK). Oligonucleotides were purchased from ThermoElectron (Ulm, Germany). Antibodies recognizing c-myc, ERK1/2, and their phosphorylated forms were from Cell Signaling Technology (Beverly, MA). Reagents for the time-resolved fluorescence resonance energy transfer (Tr-FRET) studies were from PerkinElmer Life and Analytical Sciences (Boston, MA).

Plasmid Construction

rMrgD Receptor. The rMrgD receptor was used as a PCR template for all rMrgD receptor constructs. For the N-terminally modified forms of the receptor, primers encoded the appropriate epitope tag sequence and introduced a stop codon after the last amino acid of the receptor sequence. For the C-terminally modified forms of the receptor, primers were designed to amplify the sequence and remove the stop codon.

FLAG-rMrgD. Sense, 5′-GATAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGAACTACACTCCTTATAGCAGCCCAGCCCCAGGT-3′; antisense, 5′-′CGCGGCTCGAGTCAGACCCAATCATTAGTACATGTGGATGGCGTCTC-3′. A HindIII site present in the sense primer and a XhoI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

c-myc-rMrgD. Sense, 5′-GATAAGCTTGCCACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGAACTACACTCCTTATAGCAGCCCAGCCCCAGGT-3′; antisense. 5′-′CGCGGCTCGAGTCAGACCCAATCATTAGTACATGTGGATGGCGTCTC-3′. A HindIII site present in the sense primer and a XhoI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

c-myc-rMrgD-eYFP. Sense, 5′-GATAAGCTTGCCACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGAACTACACTCCTTATAGCAGCCCAGCCCCAGGT-3′; antisense, 5′-CGGCCGGTACCGACCCCATCATTAGTACATGTGGATGGCGTCTCCCTG-3′. A HindIII site present in the sense primer and a KpnI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 upstream of and in frame with enhanced yellow fluorescent protein (eYFP) ligated between KpnI and NotI.

rMrgE Receptor. The rat MrgE receptor was used as a PCR template for all rMrgE receptor constructs. For the N-terminally modified forms of the receptor, primers encoded the appropriate epitope tag sequence and introduced a stop codon after the last amino acid of the receptor sequence. For the C-terminally modified forms of the receptor, an antisense primer was designed to remove the stop codon.

FLAG-rMrgE. Sense, 5′-GATAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGTCCCTGAGAGTGCACACGCATTCTCCCAGCACC-3′; antisense, 5′-CGCGGCTCGAGTTAGACAGTCATGTCCACAAGTCCCCCTTGGGAAGC-3′. A HindIII site present in the sense primer and a XhoI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3.

c-myc-rMrgE. Sense, 5′-GATAAGCTTGCCACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGTCCCTGAGAGTGCACACGCATTCTCCCAGCACC-3′; antisense, 5′-CGCGGCTCGAGTTAGACAGTCATGTCCACAAGTCCCCCTTGGGAAGC-3′. A HindIII site present in the sense primer and a XhoI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

FLAG-rMrgE-eYFP. Sense, 5′-GATAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGTCCCTGAGAGTGCACACGCATTCTCCCAGCACC-3′; antisense, 5′CTAATGCGGCCGCTGACAGTCATGTCCACAAGTCCCCCTTGGGAAGCCTCT-3′. A HindIII site present in the sense primer and a NotI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 upstream and in frame with eYFP ligated between NotI and XhoI.

rMrgE-eYFP. Sense, 5′-GATAAGCTTGCCACCATGTCCCTGAGAGTGCACACGCATTCTCCCAGCACC-3′; antisense, 5′CTAATGCGGCCGCTGACAGTCATGTCCACAAGTCCCCCTTGGGAAGCCTCT-3′. A HindIII site present in the sense primer and a NotI site present in the antisense primer are underlined, and the amplified fragment was digested and ligated into pcDNA3 upstream and in frame with eYFP ligated between NotI and XhoI.

Generation of Stable Flp-In T-REx HEK293 Cells

Cells were maintained in Dulbecco's modified Eagle's medium without sodium pyruvate, 4500 mg/liter glucose, and L-glutamine supplemented with 10% (v/v) fetal calf serum, 1% antibiotic mixture, and 10 μg/ml blasticidin at 37°C in a humidified atmosphere of air/CO_2 (19:1). To generate Flp-In T-REx HEK293 cells able to inducibly express c-myc-rMrgD, c-myc-rMrgE, FLAG- rMrgE-eYFP, or FLAG-rMrgD receptors, the cells were transfected with a mixture containing the desired receptor cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using LipofectAMINE (Invitrogen) according to the manufacturers' instructions. After 48 h, the medium was changed to medium supplemented with 200 μg/ml hygromycin B to initiate selection of stably transfected cells. To constitutively stably coexpress a second receptor of the rMrg family in inducible cell lines, the appropriate cells were further transfected with the desired receptor cDNA in pcDNA3 as described above, and resistant cells were selected in the presence of 1 mg/ml G418. Resistant clones were screened for receptor expression by Western blotting. Cells were treated with 1 μg/ml doxycycline 24 to 96 h before assays to induce expression of receptors cloned into the Flp-In locus.

Receptor Internalization

Monolayers of cells in 96-well plates were induced with 1 μg/ml doxycycline and incubated with growth medium containing vehicle or varying concentrations of β-alanine for 30 min at 37°C. Afterward, cell surface receptors were labeled with anti-c-myc antibody (1:500) in growth medium for 30 min at 30°C. The cells were washed once with 20 mM HEPES/Dulbecco's modified Eagle's medium and then incubated for another 30 min at 37°C in growth medium supplemented with anti-rabbit horseradish-peroxidase-conjugated IgG as secondary antibody and 1 μM Hoechst nuclear stain (Sigma) to determine cell number. The cells were then washed twice with phos-

phate-buffered saline and incubated with SureBlue (Insight Biotechnology, Wembley, Middlesex, UK) for 5 min in the dark at room temperature, and absorbance was read at 620 nm in a Victor^2 plate reader (PerkinElmer Life and Analytical Sciences). Receptor internalization was determined as loss of cell surface receptors in agonist-treated cells.

Immunostaining for N-Terminal c-myc-Tagged rMrgD and rMrgE Receptors

Immunostaining was performed essentially according to the method of Cao et al. (1999). Cells were plated on to coverslips and induced with 1 μg/ml doxycycline. After 24 to 72 h, the medium was changed for 20 mM HEPES/Dulbecco's modified Eagle's medium containing the anti-c-myc antibody diluted 1:100 and incubated for 40 min at 37°C in 5% CO_2. Where required, 20 mM HEPES/Dulbecco's modified Eagle's medium containing the desired concentration of agonist was added and incubated for 30 min at 37°C in 5% CO_2. Coverslips were washed three times with phosphate-buffered saline, and then cells fixed with 4% paraformaldehyde in phosphate-buffered saline/5% sucrose for 10 min at room temperature followed by three more phosphate-buffered saline washes. Cells were then permeabilized in 0.15% Triton X-100/3% nonfat milk/phosphate-buffered saline for 10 min at room temperature. The coverslips were subsequently incubated with an Alexa 594-labeled goat anti-mouse secondary antibody (Invitrogen) at a dilution of 1:400 (1–4 mg/ml), upside down on Nescofilm, for 1 h at room temperature, then washed twice in 0.15% Triton X-100/3% nonfat milk/phosphate-buffered saline and three times with phosphate-buffered saline. Finally, coverslips were mounted onto microscope slides with 40% glycerol in phosphate-buffered saline.

Confocal Laser-Scanning Microscopy

Cells were observed using a confocal laser-scanning microscope (LSM 5 PA; Zeiss, Jena, Germany) using a Zeiss Plan-Apo 63 × 1.40 numerical aperture oil immersion objective, with a pinhole of 20 and electronic zoom 1 or 2.5 (Milasta et al., 2005). eYFP was excited using an argon laser at 488 nm and detected with a band-pass filter at 505 to 530 nm. The Alexa 594 label was excited using a helium/neon laser at 543 nm and detected with a long-pass filter at 560 nm. The images were analyzed with MetaMorph software. For the receptor internalization studies, fixed cells were used. Cells on glass coverslips were washed with phosphate-buffered saline and fixed for 10 min at room temperature using 4% paraformaldehyde in phosphate-buffered saline/5% sucrose, pH 7.4. After three washes with phosphate-buffered saline, coverslips were mounted on to microscope slides with 40% glycerol in phosphate-buffered saline.

Coimmunoprecipitation Studies

Cells were harvested 24 to 72 h after induction with 1 μg/ml doxycycline and resuspended in RIPA buffer (50 mM HEPES, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM NaF, 5 mM EDTA, 0.1 mM $NaPO_4$, and 5% ethylene glycol). The cell pellet was placed on a rotating wheel for 1 h at 4°C. Samples were then centrifuged for 1 h at 100,000*g* at 4°C, and the supernatant was transferred to a fresh tube containing 200 μl of RIPA and 50 μl of Protein G beads (Sigma) to preclear the samples. After incubation on a rotating wheel for 1 h at 4°C, the samples were re-centrifuged at 20,800*g* at 4°C for 1 min, and the protein concentration of the supernatant was determined. Samples containing equal amounts of protein were incubated overnight with 40 μl of Protein G beads and 5 μg of M2 anti-FLAG antibody (Sigma) at 4°C on a rotating wheel and fractions reserved to monitor protein expression in the cell lysates. Samples were centrifuged at 20,800*g* for 1 min at 4°C and the Protein G beads washed three times with RIPA buffer. After addition of 2× reducing loading buffer and heating to 85°C for 4 min, both immunoprecipitated samples and cell lysate controls were resolved by SDS-PAGE using precast 4 to 12% acrylamide Novex Bis-tris gels (Invitrogen BV). Proteins were transferred onto PVDF membrane. These membranes were incubated in 5% (w/v) low fat milk, 0.1% Tween 20/Tris-buffered saline (TBS) (v/v) solution at room temperature on a rotating shaker for 1 h and then with primary antibody overnight in 5% (w/v) low-fat milk, 0.1% Tween 20/TBS (v/v) solution at 4°C. The membrane was washed three times in TBS/0.1% Tween 20 before addition of secondary antibody. After further washes, the membrane was subsequently developed using ECL solution (Pierce Chemical, Cramlington, Northumberland, UK).

ERK1/2 Phosphorylation and Immunoblots

Cells were grown in six-well plates and serum-starved overnight before stimulation with ligands as indicated. Cells were then placed on ice, washed twice with ice-cold phosphate-buffered saline, and lysed in RIPA buffer. After 1 h at 4°C, the lysates were centrifuged for 15 min at 20,800*g* at 4°C to remove the insoluble material. The samples were mixed with 2× reducing loading buffer and heated for 3 min at 95°C. ERK1/2 phosphorylation was detected by protein immunoblotting using phospho-ERK1/2-specific antibodies and anti-rabbit horseradish peroxidase-conjugated IgG as secondary antibody for immunodetection. After visualizing the level of ERK1/2 phosphorylation, the PVDF membranes were stripped and reprobed using the anti-ERK1/2 antibody.

$[Ca^{2+}]_i$ Imaging

Cells induced or not to express receptors were loaded with the Ca^{2+}-sensitive dye Fura-2 (Sigma) by incubation (15–20 min; 37°C) under reduced light in Dulbecco's modified Eagle's medium containing the dye's membrane-permeant acetoxymethyl ester form (1.5 μM). Details of the imaging studies and their analysis have been described previously (Liu et al., 2002).

Time-Resolved Fluorescence Resonance Energy Transfer

Tr-FRET was performed using a combination of an Eu^{3+}-labeled anti-c-myc antibody, as a long-lived energy donor, and allophycocyanin-labeled anti-FLAG antibody as a potential energy acceptor (McVey et al., 2001; Wilson et al., 2005) to cells constitutively expressing c-myc-rMrgE that were induced or not to express FLAG-rMrgD.

Gene Expression Analysis

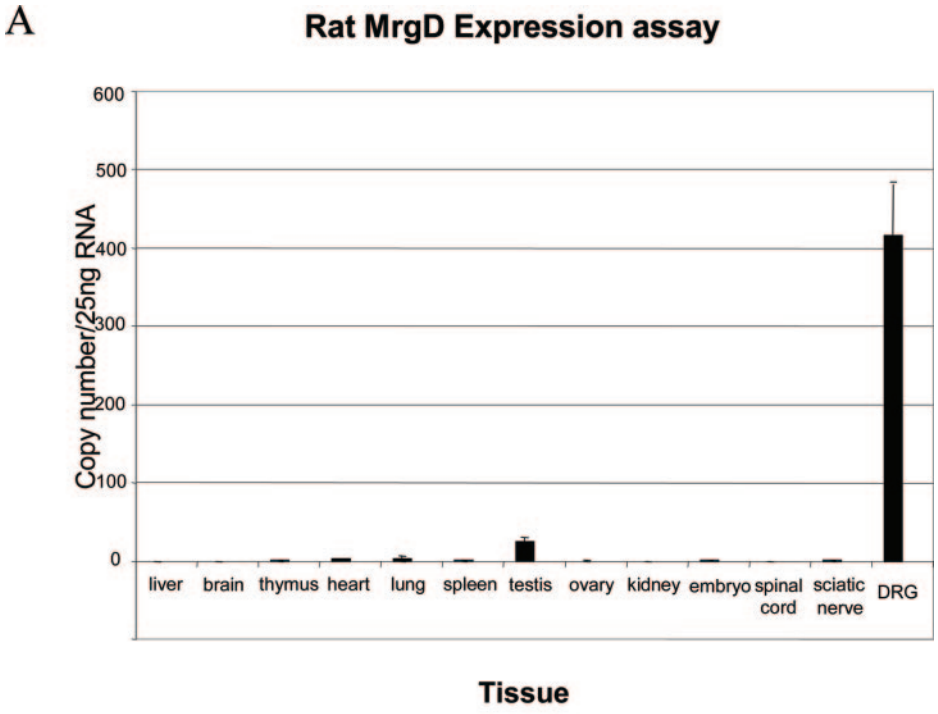
RNA (100 ng) from each sample of the rat total RNA panel (Clontech, Mountain View, CA) was reverse-transcribed in triplicate using a GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. A quarter of this cDNA (25 ng) was used as a template for quantitative PCR using the 7900HT sequence detection system (Applied Biosystems). Absolute quantitation was achieved by means of a 5-log ($10–10^5$ C-value) standard curve of rat genomic DNA (Clontech), assuming that one C-value (2.65 pg) contains one copy of the target gene. The reaction was run according to the manufacturer's instructions for absolute quantitation, with primer conditions determined from primer and probe optimization studies performed per Applied Biosystems protocol. Forward primer (300 nM) (CAGCCTCGGCGGCTCTA), reverse primer (900 nM) (CCAACGGCAGAGAACAGGTAAG), and dual labeled probe (175 nM) (5′-FAM-TGGTCATCCTGACTTCCGTCCT-TGTCTTC-TAMRA-3′) were used for rMrgD. Identical primer concentrations were also used for rMrgE (forward, TGGCACACACCC-CTCTACTTCT; reverse, AGGCTTGGCCGCACTGT) with probe (150 nM) (5′–5-carboxyfluorescein-TCACTTCAGCTTCTTCATGGCCAG-TGTG-5-carboxytetramethylrhodamine–3′). Error bars represent the S.D. of the triplicate samples.

Results

Distribution Pattern of rMrgD and rMrgE Receptor Messenger RNA. The distribution of mRNA encoding the MrgD and MrgE receptors in rat tissues was assessed via

quantitative reverse transcription-polymerase chain reaction after isolation of RNA. As anticipated from previous work (Zhang et al., 2005), a substantial amount of mRNA encoding each of MrgD and MrgE was present in dorsal root ganglion tissue. By contrast, levels of mRNA encoding MrgD were essentially undetectable in all other tissues examined, apart from testis. MrgE mRNA could also be detected in a crude brain sample, spinal cord, and sciatic nerve but at levels no more than 20% of that in dorsal root ganglion (Fig. 1). Such results confirmed the expression of both MrgD and MrgE mRNA, and thus presumably protein, in dorsal root ganglia.

Homodimeric Interactions of rMrgD and rMrgE Receptors. It is becoming widely accepted that GPCRs are able to form homodimers/oligomers and that this may be impor-



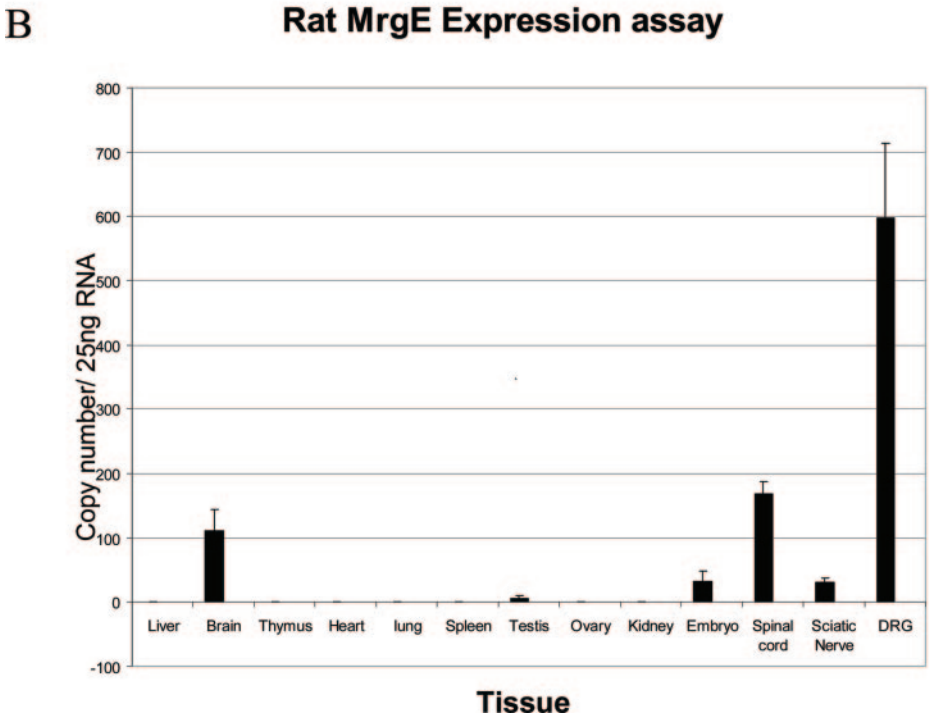


Fig. 1. Coexpression of MrgD and MrgE receptor mRNA in rat dorsal root ganglia. RNA from various rat tissues was used for quantitative reverse transcriptase-PCR as detailed under *Materials and Methods* to detect mRNA encoding MrgD (A) or MrgE (B). Only dorsal root ganglia contained high copy number of mRNA for each receptor. Error bars represent the S.D. of three samples.

tant for cellular trafficking and function. Because this has not been reported previously for any member of the Mrg receptor family, we expressed N-terminally FLAG and c-myc tagged forms of rat (r)MrgD either individually or in combination in HEK293 cells. Samples were immunoprecipitated with an anti-FLAG antibody, resolved by SDS-PAGE, and then immunoblotted to detect the presence of c-myc reactive polypeptides in these immunoprecipitates. Only after coexpression of FLAG and c-myc rMrgD was a c-myc reactive polypeptide of some 33 kDa present in the anti-FLAG immunoprecipitates (Fig. 2). This is consistent with the two coexpressed forms of rMrgD being present within a dimeric/oligomeric complex. Equivalent studies with FLAG and c-myc-tagged forms of rMrgE also resulted in the presence of a c-myc-reactive 28-kDa polypeptide in anti-FLAG immunoprecipitates only when the two forms of rMrgE were coexpressed (Fig. 2).

Stable Expression of rMrgD and rMrgE Receptors in HEK293 Cells. To investigate function and potential physical interactions between the rMrgD and rMrgE receptors, we used HEK293 Flp-In T-REx cells with the capacity to stably express either receptor alone or both receptors together. In all the single receptor-expressing cell lines produced for these studies, the receptor of interest was cloned into the Flp-In locus to allow inducible expression under the control of a tetracycline-on promoter. Clones for expression of each of N-terminally FLAG- and c-myc-tagged forms of rMrgD and N-terminally c-myc-tagged rMrgE as well as rMrgE C-terminally tagged with eYFP were generated. Immunoblots of cell lysates using anti-FLAG and anti-c-myc antibodies confirmed expression of the anticipated receptor polypeptides in a manner that was entirely dependent upon addition of doxycycline as inducing agent (Fig. 3A). In this system, c-myc-rMrgE migrated through SDS-PAGE as an apparent 28-kDa polypeptide with some evidence of heterogeneity, which may reflect differential glycosylation (Fig. 3A), whereas both FLAG- and c-myc-tagged forms of rMrgD migrated predominantly as polypeptides of some 33-kDa polypeptides (Fig. 3A).

Clones capable of coexpressing rMrgD and rMrgE were subsequently obtained by transfection of the above cell lines using conventional transfection and selection with a second antibiotic resistance marker. This resulted in the production of clones in which one receptor was expressed constitutively and the second could be produced upon treatment with doxycycline. As shown in Fig. 3, B and C, in appropriate clones, rMrgE tagged at the C terminus with eYFP or N-terminally c-myc-tagged rMrgE receptors could be detected in whole-cell lysates in the absence of doxycycline treatment (Fig. 3, B and C), whereas differentially tagged forms of rMrgD were only

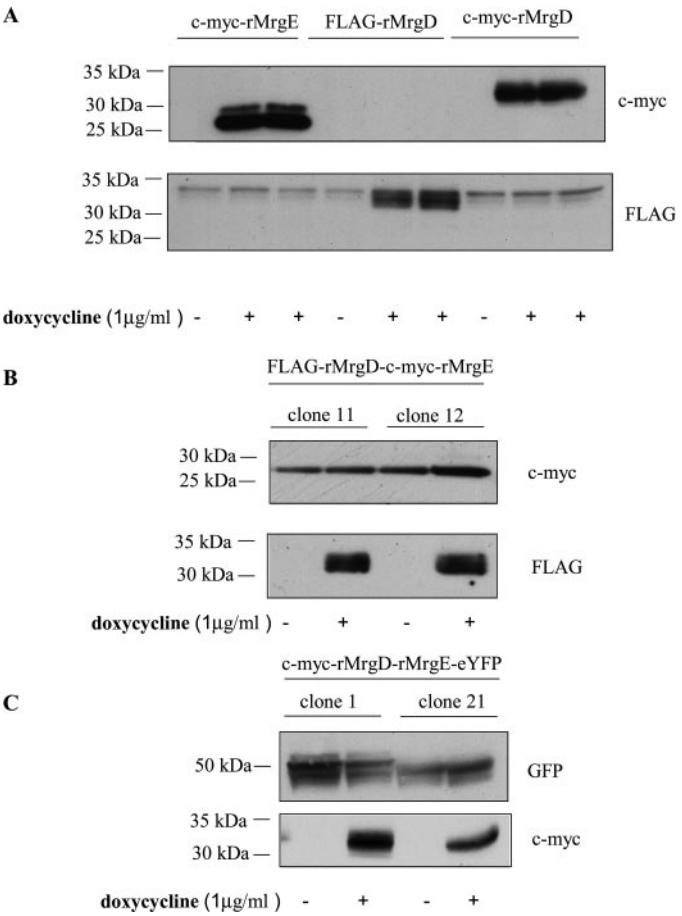


Fig. 3. Constitutive and inducible expression of rMrgD and rMrgE receptor constructs in Flp-In T-REx HEK293 cells. A, Flp-In T-REx HEK293 cell lines were generated to allow inducible expression of N-terminally c-myc-tagged-rMrgE, FLAG-tagged rMrgD, or c-myc-tagged-rMrgD. Cells were grown with or without 1 μg/ml doxycycline for 24 h. Whole-cell lysates were prepared and samples containing 20 μg of protein were resolved by SDS-PAGE, transferred onto a PVDF membrane and immunoblotted with anti-c-myc (top) or anti-FLAG (bottom) antibodies. B and C, cells as in A, with the capacity to inducibly express FLAG- (B) or c-myc- (C) tagged rMrgD, were further transfected to stably and constitutively express c-myc-rMrgE (B) or FLAG-rMrgE-eYFP (clone 1) or rMrgE-eYFP (clone 21) (C). After growth with or without 1 μg/ml doxycycline for 24 h whole-cell lysates were prepared and the individual receptors detected as in A using anti-c-myc, anti-FLAG, or anti-GFP antibodies.

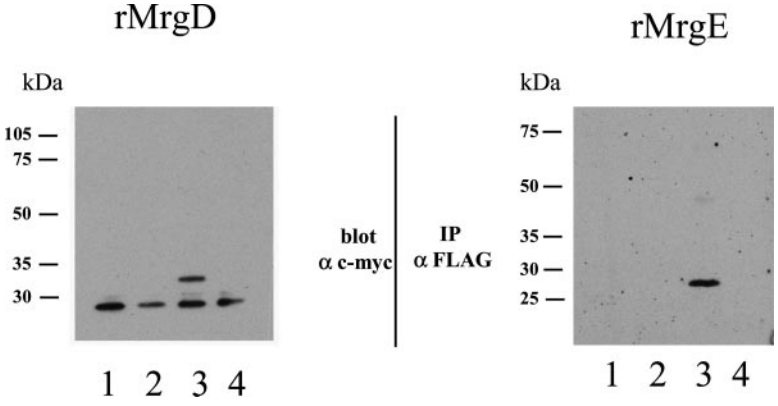


Fig. 2. rMrgD and rMrgE form constitutive homodimeric/oligomeric complexes. FLAG (1 and 3) and c-myc (2 and 3)-tagged forms of rMrgD (left) or rMrgE (right) were expressed transiently in HEK293 cells either individually (1–2) or in combination (3). In 4, samples individually expressing FLAG or c-myc-tagged receptors were mixed. Anti-FLAG antibody was used to immunoprecipitate samples and these were resolved by SDS-PAGE and subsequently immunoblotted with anti-c-myc.

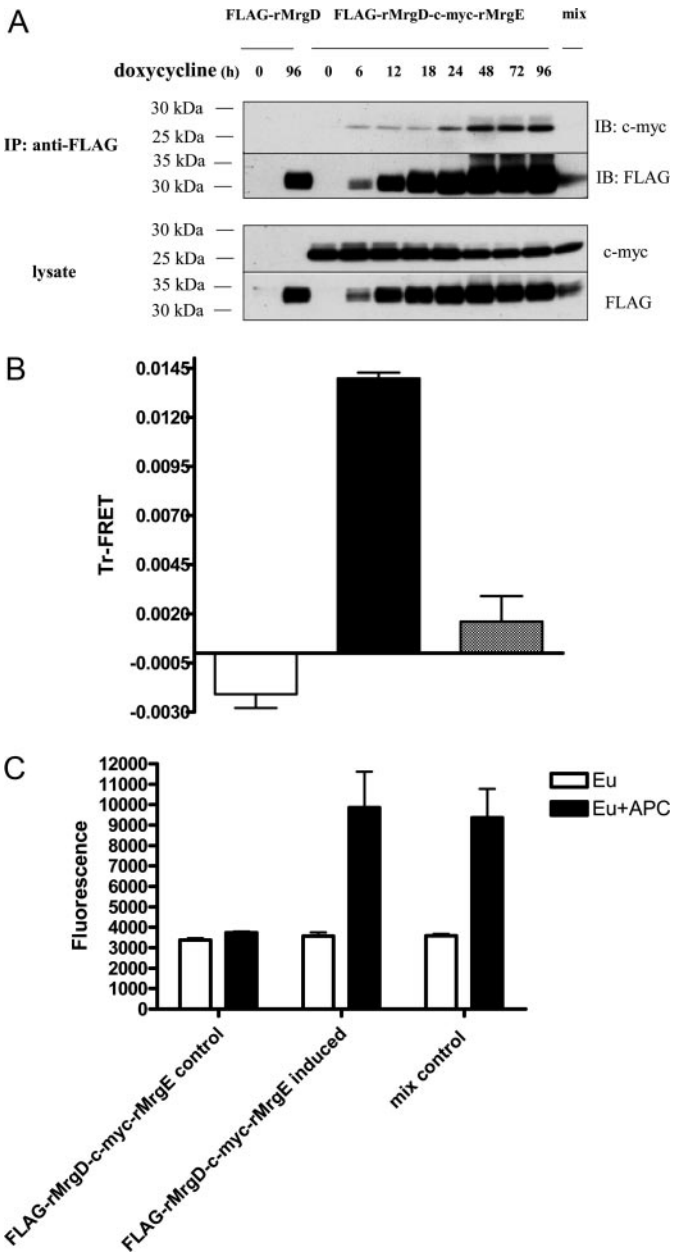


Fig. 4. Constitutive heterodimerization between coexpressed rMrgD and rMrgE receptors revealed by coimmunoprecipitation and Tr-FRET. A, Flp-In T-REx HEK293 cells either harboring FLAG-rMrgD at the inducible locus only or constitutively expressing c-myc-rMrgE and harboring FLAG-rMrgD at the inducible locus were induced with 1 μg/ml doxycycline for varying times. In the "mix" samples, lysates from FLAG-rMrgD induced for 96 h and of uninduced FLAG-rMrgD-c-myc-rMrgE cells were mixed before analysis. Top, cell lysates were immunoprecipitated with anti-FLAG antibody, and samples were resolved by SDS-PAGE and then immunoblotted with either anti-c-myc or anti-FLAG antibodies. Bottom, Western blot analysis of cell lysates using anti-FLAG and anti-c-myc antibodies demonstrated maintained levels of c-myc-rMrgE over time and the time course of induction of FLAG-rMrgD. B, Flp-In T-REx HEK293 cells constitutively expressing c-myc-rMrgE and harboring FLAG-rMrgD at the inducible locus were treated with (black bars) or without (open bars) 1 μg/ml doxycycline for 24 h. A combination of Eu^{3+}-labeled anti-c-myc antibody and APC-labeled anti-FLAG antibody was added and Tr-FRET measured as described under *Materials and Methods*. The pair of antibodies was also added to a mixture of cells induced to express either c-myc-rMrgE or FLAG-rMrgD (gray bar). Data represent means ± S.E.M. from three independent experiments. C, Flp-In T-REx HEK293 cells constitutively expressing c-myc-rMrgE and harboring FLAG-rMrgD at the inducible locus were induced (induced) or not (control) to express FLAG-rMrgD. A mix control was generated as in B. APC-labeled anti-FLAG antibody was added; after washing, fluorescence corresponding to APC bound to the cells was measured. Equivalent signal above background was present in the induced and mixed control, confirming that the lack of Tr-FRET in the mix control in B did not reflect poor induction of expression of FLAG-rMrgD. Data represent means ± S.E.M. from three independent experiments.

present after treatment of the cells with doxycycline. Induced expression of the various rMrgD receptor constructs did not modulate the levels of constitutively expressed rMrgE receptors because the various forms of rMrgE could be detected at similar levels in cell lysates with and without treatment with doxycycline to induce expression of rMrgD (Fig. 3, B and C). Multiple clones had equivalent characteristics (Fig. 3 and data not shown).

In cells expressing c-myc-rMrgE constitutively, FLAG-rMrgD was expressed in a time-dependent manner after addition of doxycycline (Fig. 4A). Although immunodetectable levels were present within 6 h, 24- to 48-h treatment with doxycycline was required to achieve maximal levels, and this was maintained for up to 72 to 96 h (Fig. 4A). In contrast, and as expected, levels of c-myc-rMrgE were largely maintained throughout this period (Fig. 4A). Coexpressed c-myc-rMrgE and FLAG-rMrgD were able to interact with one another, because immunoprecipitation of FLAG-rMrgD resulted in coimmunoprecipitation of c-myc-rMrgE (Fig. 4A). Coimmunoprecipitation of c-myc-rMrgE in anti-FLAG immunoprecipitates only occurred with detectable expression of FLAG-rMrgD, and the amount of coimmunoprecipitation of c-myc-rMrgE mirrored the time course of induction and extent of production of FLAG-rMrgD (Fig. 4A). In induced cells expressing only FLAG-rMrgD, this receptor was expressed to levels similar to those in the cells able to coexpress FLAG-rMrgD and c-myc-rMrgE; however, although the anti-FLAG antibody was effective in immunoprecipitating FLAG-rMrgD from lysates of these cells, no c-myc reactive polypeptides were coimmunoprecipitated (Fig. 4A). Confirmation of the specificity and requirement for coexpression to allow coimmunoprecipitation of c-myc-rMrgE and FLAG-rMrgD was obtained by mixing cell lysates of induced cells expressing only FLAG-rMrgD with those from cells constitutively expressing c-myc-rMrgE but not induced to express FLAG-rMrgD. No coimmunoprecipitation of c-myc-rMrgE was obtained from such mixtures of lysates, although FLAG-rMrgD reactivity was present in the anti-FLAG immunoprecipitates (Fig. 4A). Delivery of FLAG-rMrgD and c-myc-rMrgE to the cell surface and interactions between FLAG-rMrgD and c-myc-rMrgE at the surface of living cells were monitored via Tr-FRET. Addition of a combination of Eu^{3+}-labeled anti-c-myc antibody as a long-lived energy donor and allophycocyanin-labeled anti-FLAG antibody as a potential resonance energy acceptor to cells constitutively expressing c-myc-rMrgE and induced to express FLAG-rMrgD resulted in a significant Tr-FRET signal (Fig. 4B). No signal was detected in these cells if FLAG-rMrgD expression was not induced (Fig. 4B), and no Tr-FRET signal was obtained when cells individually expressing c-myc-rMrgE or FLAG-rMrgD were mixed and then exposed to the combination of antibodies (Fig. 4B). Controls that simply measured fluorescence of the allophycocyanin-labeled anti-FLAG antibody bound to cells demonstrated similar levels of cell surface FLAG-rMrgD receptors in the cells coexpressing the two receptors and in the mixed cell populations (Fig. 4C).

Internalization of rMrgD but Not rMrgE in Response to β-Alanine. β-Alanine has been described as an agonist for MrgD that is able to cause internalization of the receptor (Shinohara et al., 2004). Cells induced to express c-myc-rMrgD only were prelabeled with anti-c-myc antibody and treated with increasing concentrations of β-alanine for 30 min. In the absence of agonist, the majority of the immunostained c-myc-rMrgD receptors detected after cell permeabilization were localized at the plasma membrane (Fig. 5A); however, a small proportion of the receptors could be detected in intracellular vesicles, suggesting that c-myc-rMrgD may partially internalize independently of agonist stimulation (Fig. 5A). β-Alanine caused substantial internalization of the c-myc-rMrgD receptor into punctate intracellular vesicles; maximum internalization was observed after treatment with 1 mM ligand (Fig. 5A). When expressed alone, c-myc-rMrgE was also expressed predominantly at the plasma membrane after induction of receptor expression (Fig. 5B). However, treatment with up to 10 mM β-alanine did not result in detectable internalization of the rMrgE receptor (Fig. 5B).

Recent reports indicate that heterodimerization of GPCRs can affect the internalization properties of the individual receptors (Jordan et al., 2001; Breit et al., 2004). To determine whether coexpression with rMrgE altered the internalization properties of rMrgD, HEK293 Flp-In T-REx cells constitutively expressing FLAG-rMrgE-eYFP were induced for 72 h with doxycycline to also express c-myc-rMrgD and to allow heterodimer formation as shown in Fig. 4. In doxycycline-induced but unstimulated cells, both receptors were expressed at the plasma membrane and displayed overlapping distributions (Fig. 6A, top). In response to β-alanine, internalization of c-myc-rMrgD receptor could be observed, whereas no extra FLAG-rMrgE-eYFP could be detected in intracellular vesicles (Fig. 6A, bottom). Unlike when c-myc-rMrgD expression was induced in the absence of rMrgE, where little cell surface staining could be observed after β-alanine treatment and most of the detectable rMrgD receptors were apparently localized in endocytic vesicles (Fig. 6A), coexpression of FLAG-rMrgE-eYFP seemed to impair β-alanine-induced sequestration of c-myc-rMrgD. This was suggested because significant amounts of immunostained c-myc-rMrgD receptor could still be detected at the plasma membrane (Fig. 6A, bottom). Such observations are entirely qualitative; thus, to quantify the extent of internalization of c-myc-rMrgD in the absence or presence of FLAG-rMrgE-

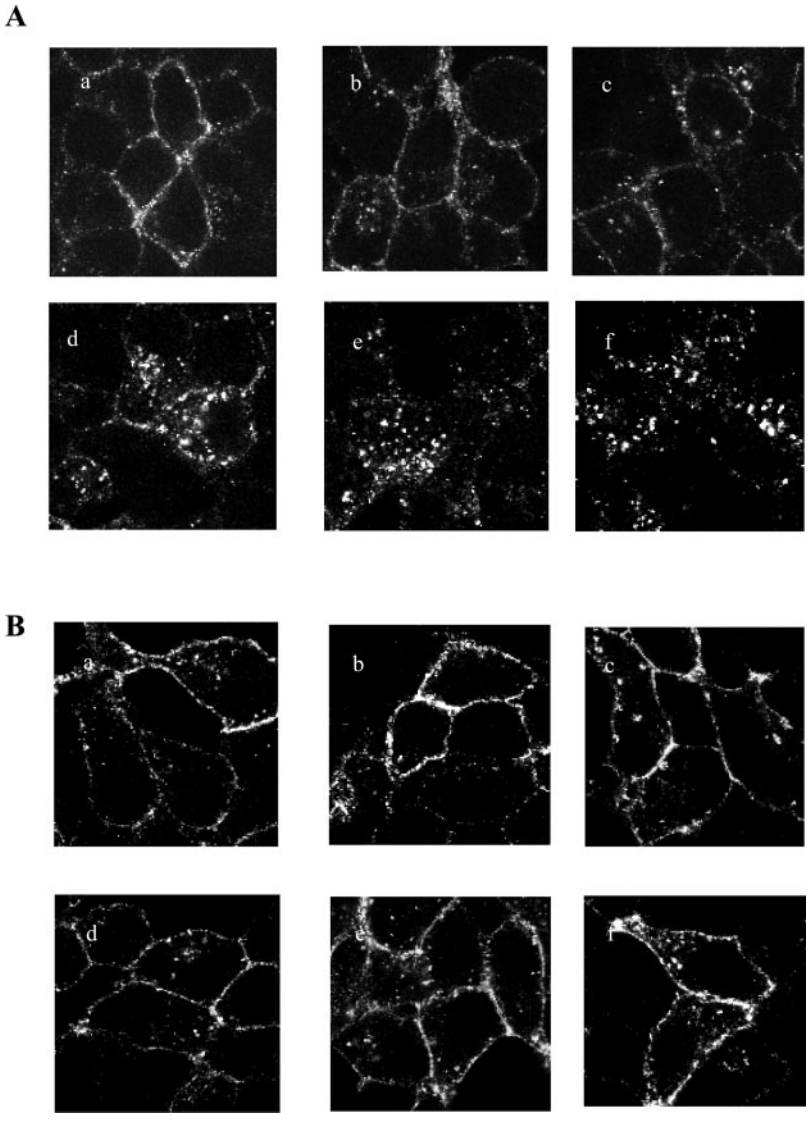


Fig. 5. Internalization of rMrgD but not rMrgE receptors in response to β-alanine. Flp-In T-REx HEK293 cell lines were induced with 1 μg/ml doxycycline for 72 h to express either c-myc-rMrgD (A) or c-myc-rMrgE (B) and then immunostained with anti-c-myc antibody before stimulation with varying concentrations of β-alanine for 30 min at 37°C. Confocal images monitoring the location of receptor-associated anti-c-myc were taken after permeabilization of untreated cells (a) and cells treated with 0.1 mM (b), 0.3 mM (c), 1 mM (d), 3 mM (e), or 10 mM (f) β-alanine. Similar results were produced in three independent experiments.

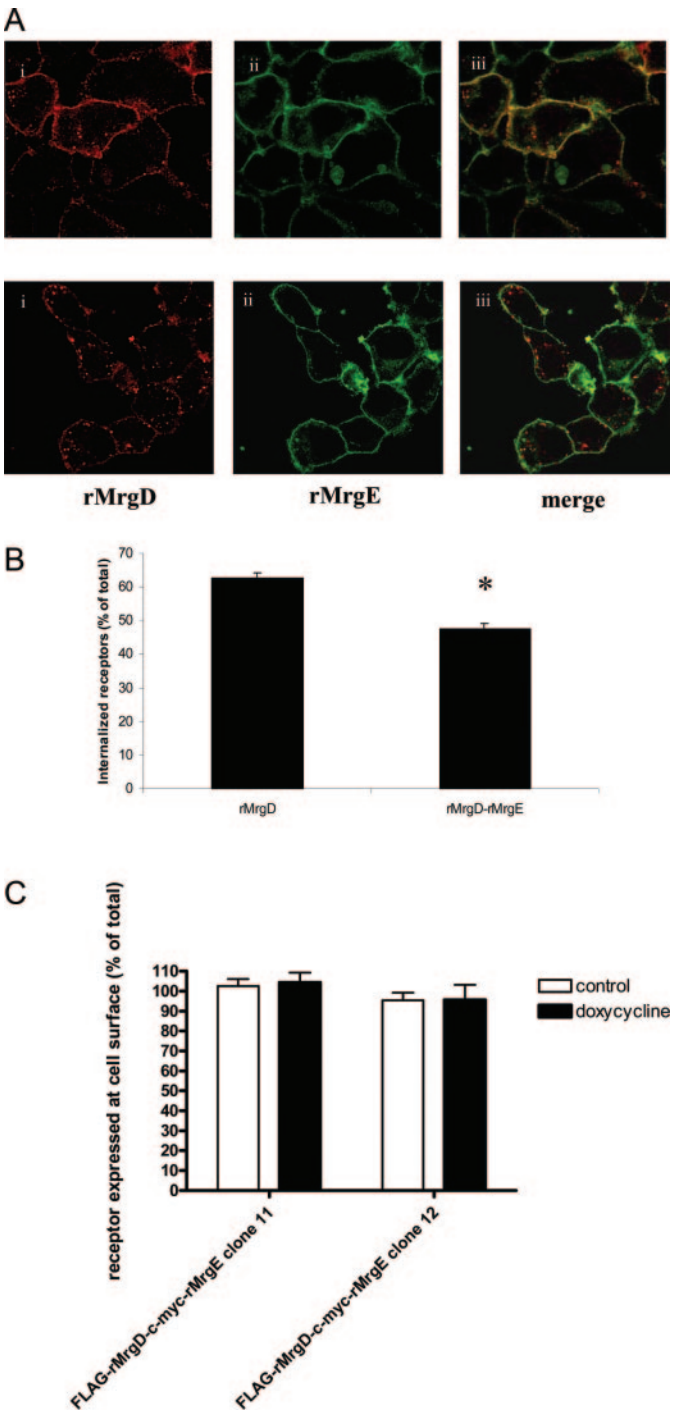


Fig. 6. Coexpression with rMrgE impairs β-alanine-induced internalization of rMrgD. A, c-myc-rMrgD receptor (i, red) expression was induced by treatment with 1 μg/ml doxycycline for 72 h in cells constitutively expressing FLAG-rMrgE-eYFP (ii, green). Cell surface c-myc-rMrgD receptors were labeled with anti-c-myc antibody and the cells treated with vehicle (top row) or 3 mM β-alanine (bottom row) for 30 min at 37°C. The overlay of the two signals (iii, top, yellow) in the absence of β-alanine was partially resolved (iii, bottom) by internalization of c-myc-rMrgD (red). B, cells as in A were untreated or stimulated with 3 mM β-alanine for 30 min at 37°C, incubated with rabbit polyclonal anti-c-myc and anti-rabbit horseradish peroxidase-conjugated antibodies, and cell surface receptor immunoreactivity was measured by colorimetry. Internalization was defined as the loss of cell surface immunoreactivity and is expressed as the percentage of the immunoreactivity present in the absence of agonist. Data represent means ± S.E.M. of four independent experiments. A lower percentage of c-myc-rMrgD was internalized when rMrgE was coexpressed ($p < 0.01$). C, two distinct clones constitutively expressing c-myc-rMrgE and harboring FLAG-rMrgD at the inducible locus were induced (filled bars) or not (open bars) to express FLAG-rMrgD. Cells were stimulated with 3 mM β-alanine for 30 min at 37°C and cell surface levels of c-myc-rMrgE measured as in B after addition of rabbit polyclonal anti-c-myc and horseradish peroxidase-conjugated anti-rabbit antibodies. Induction of FLAG-rMrgD expression did not result in internalization of c-myc-rMrgE in response to β-alanine. Data represent means ± S.E.M. of four independent experiments.

eYFP, enzyme-linked immunosorbent assays were performed. Cells induced to express c-myc-rMrgD with or without constitutive expression of FLAG-rMrgE-eYFP were treated with or without β-alanine for 30 min and then immunostained with anti-c-myc-antibody and a horseradish peroxidase secondary antibody to allow detection of c-myc-rMrgD receptors at the cell surface. The extent of rMrgD receptor endocytosis was reduced significantly ($p < 0.01$) in the presence of rMrgE, reaching only 48 ± 2% compared with 63 ± 2% in cells expressing only c-myc-rMrgD (Fig. 5B). These results demonstrate that coexpression of rMrgE impairs internalization of rMrgD in response to β-alanine and are consistent with the concept that the rMrgD-rMrgE heterodimer is either unable to internalize in response to agonist stimulation or is less able than the rMrgD homodimer. By contrast, and in agreement with the confocal images, enzyme-linked immunosorbent assays confirmed no significant internalization of FLAG-rMrgE-eYFP in response to β-alanine whether FLAG-rMrgE-eYFP was expressed alone or when c-myc-rMrgD was coexpressed by treatment with doxycycline (Fig. 6C).

***β*-Alanine Is an Agonist at rMrgD but Not rMrgE.** The observation that treatment with β-alanine caused sequestration of rMrgD but not of rMrgE from the plasma membrane to intracellular vesicles did not exclude the possibility that rMrgE might generate downstream signals in response to β-alanine because ligand-stimulated receptor internalization is not an infallible surrogate marker for agonism of downstream signaling events (Roettger et al., 1997; Whistler et al., 2002). To explore signal transducing effects of β-alanine at the rMrgD and rMrgE receptors, β-alanine (100 μM)-mediated phosphorylation of the ERK1/2 mitogen-activated protein kinases was examined initially in cells induced to express only either rMrgD or rMrgE receptors. In cells expressing only rMrgD, β-alanine stimulated ERK1/2 phosphorylation in a transient manner; maximal effects were observed after 5 min, and signal returned to basal levels within 15 min (Fig. 7A). No effect of β-alanine was observed in these cells if rMrgD expression had not been induced by treatment with doxycycline (data not shown). By contrast, β-alanine was unable to cause phosphorylation of ERK1/2 in cells induced to express rMrgE (Fig. 7B). β-Alanine was also able to cause phosphorylation of ERK1/2 in cells that constitutively expressed rMrgE and in which coexpression of rMrgD was induced by treatment with doxycycline. The time course of ERK1/2 phosphorylation was similar to that observed in cells expressing only rMrgD (Fig. 7C). To further examine potential effects of rMrgD-rMrgE receptor heterodimerization on ERK1/2 activation, β-alanine-concentration-response curves were performed. The maximal response of ERK1/2 phosphorylation in cells expressing only rMrgD was observed with addition of 1 mM β-alanine (Fig. 8A), a concentration of ligand similar to that necessary to cause maximal receptor internalization (Fig. 5A). To ascertain that

this rMrgD receptor response was specific for β-alanine and was not caused by a nonspecific effect reflecting the high concentration of β-alanine required, rMrgD receptor-expressing cells were treated with the same concentrations of L-alanine. No ERK1/2 activation could be detected (Fig. 8B). Cells expressing either rMrgD or rMrgE alone or coexpressing both receptors were treated with concentrations of β-alanine ranging from 0.01 to 10 mM to examine whether the formation of rMrgD-rMrgE receptor heterodimers has an effect on the sensitivity of ligand-induced ERK1/2 phosphorylation.

It is noteworthy that a significant ($p < 0.01$) 2.5-fold increase in potency of β-alanine was observed in cells coexpressing rMrgE and rMrgD (Fig. 9), although cells expressing rMrgE alone did not respond to β-alanine at any concentration tested (Fig. 9). To extend these observations, we also measured changes in $[Ca^{2+}]_i$ in response to β-alanine treatment of rMrgD and rMrgE receptor expressing cells. Stimulation of single rMrgD receptor expressing cells induced a rapid and transient elevation of $[Ca^{2+}]_i$, whereas no elevation in $[Ca^{2+}]_i$ could be observed after addition of β-alanine to uninduced cells harboring the rMrgD receptor at the Flp-In locus or cells induced to express rMrgE (Fig. 10). The capacity of β-alanine to elevate $[Ca^{2+}]_i$ in cells in which induction of rMrgD expression was maintained for varying times before analysis showed both a reduction in the maximal signal and a slower kinetic of onset in cells that had been expressing rMrgD for 72 h compared with those expressing this receptor for 24 h (Fig. 11). It is intriguing that this time-dependent loss of rMrgD-mediated function of β-alanine was completely absent in cells in which rMrgD was induced for similar periods but in the presence of constitutive expression of rMrgE (Fig. 11).

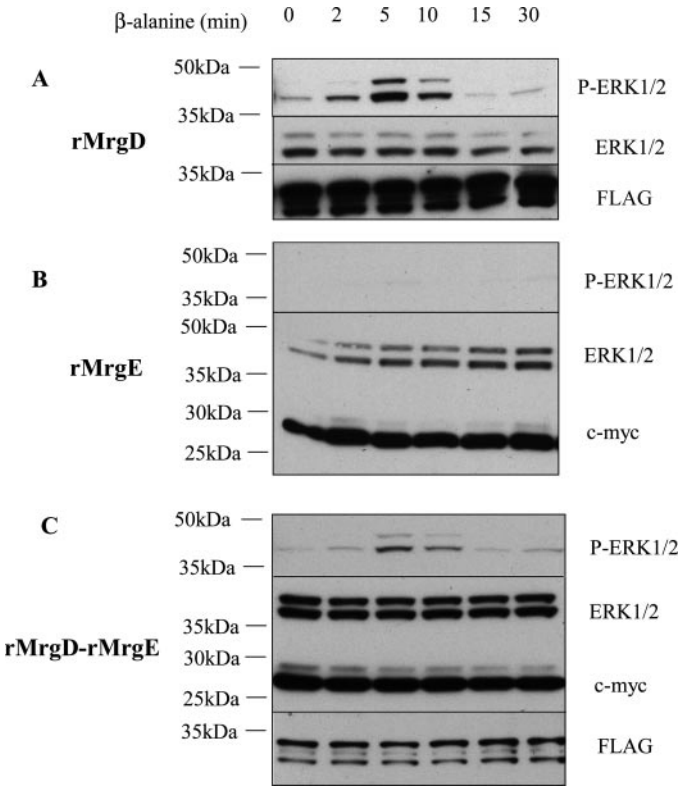


Fig. 7. β-Alanine stimulates phosphorylation of ERK1/2 via rMrgD but not rMrgE. Cells were induced to express FLAG-rMrgD (A), c-myc-rMrgE (B), or FLAG-rMrgD (C) in the presence of constitutively expressed c-myc-rMrgE. After overnight serum starvation cells were stimulated with 100 μM β-alanine for the indicated times at 37°C. ERK1/2 phosphorylation was then detected using phosphospecific anti-ERK1/2 antibodies (P-ERK1/2). Expression levels of ERK1/2 were monitored using antibodies directed against the total population of ERK1/2 (ERK1/2). Expression of FLAG-rMrgD and c-myc-rMrgE was also monitored. Similar results were produced in three separate experiments.

Discussion

Most members of the Mrg family of GPCRs are expressed predominantly or exclusively in dorsal root ganglion neurons that are key for perception of pain (Dong et al., 2001; Lembo et al., 2002). The family is substantial in number in both primates and rodents (Dong et al., 2001; Lembo et al., 2002; Zylka et al., 2003). However, although marked expansion (Zylka et al., 2003) of the number and adaptive evolution (Choi and Lahn, 2003) of genes encoding members of the MrgA and MrgC subfamilies in mice (Zylka et al., 2003) has raised questions as to the identity of orthologs in other species, including man, there seem to be only single genes encoding the MrgD and MrgE receptors in different species, and these are coexpressed in individual dorsal root ganglion cells (Zhang et al., 2005). A number of reports have indicated the ability of a range of peptide ligands to activate various Mrg family receptors from different species (Dong et al., 2001; Han et al., 2002; Lembo et al., 2002; Robas et al., 2003; Grazzini et al., 2004; Kamohara et al., 2005). However, a small number of Mrg family receptors have been shown to respond to more simple, nonpeptide ligands (Bender et al., 2002; Shinohara et al., 2004). MrgD (also named TRG7) from various species is activated by β-alanine (Shinohara et al., 2004); to date, however, the closely related MrgE remains an orphan GPCR.

In recent years, the concept that GPCRs can form in trans-

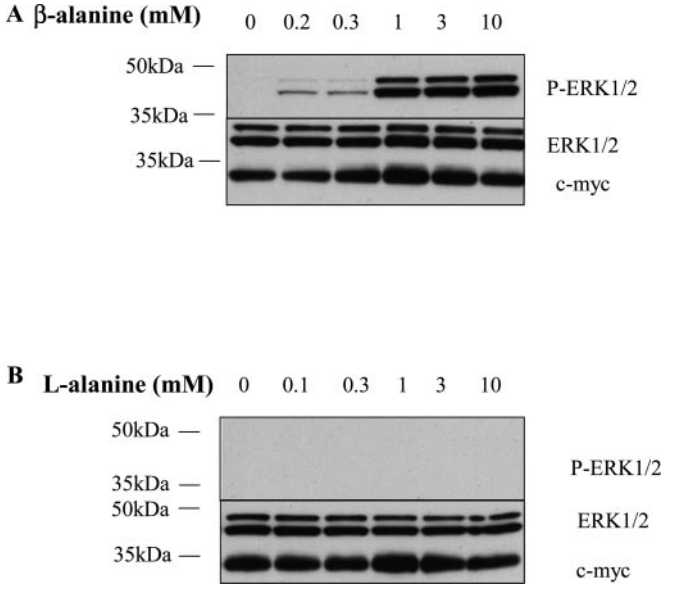


Fig. 8. Selectivity of β-alanine-induced ERK1/2 phosphorylation. Flp-In T-REx HEK293 cells were treated with 1 μg/ml doxycycline to induce expression of c-myc-rMrgD. Twenty-four hours later, the cells were serum-starved and then treated with varying concentrations of β-alanine (A) or L-alanine (B) for 5 min at 37°C. Phosphorylation of ERK1/2 was evaluated by immunoblot analyses using antiphosphospecific ERK1/2 antibodies (P-ERK1/2). Total ERK1/2 levels (ERK1/2) and the presence of the receptors (c-myc) were monitored as in Fig. 7.

fected cell systems, and exist in physiological settings, as homodimers or homo-oligomers (Angers et al., 2002; Milligan et al., 2003; Breitwieser, 2004; Milligan, 2004) has been tested widely using approaches that range from coimmunoprecipitation of differentially epitope-tagged polypeptides to atomic force microscopy (Milligan and Bouvier, 2005). In many cases, such interactions seem to occur during protein synthesis and to be important for delivery of functional receptors to the surface of cells (Terrillon et al., 2003; Salahpour et al., 2004; Bulenger et al., 2005). It has been claimed that greater than 90% of the entire family of nonchemosensory GPCRs is expressed to some level in the central nervous system (Vassilatis et al., 2003), and gene chip analysis of GPCR expression in regions of brain, including key small nuclei, suggests that many GPCRs are likely to be coexpressed in specific neurons (Hakak et al., 2003). As such, demonstrations that certain GPCR pairs can form heterodimers/oligomers (George et al., 2002; Milligan, 2004; Bulenger et al., 2005) as well as homodimers/oligomers, even in physiological settings (AbdAlla et al., 2001; Kostenis et al., 2005), have attracted considerable attention. Key issues that are currently being addressed include whether such heterodimers display distinct pharmacology and function and, if so, whether they might provide novel sets of targets for therapeutic intervention in disease (Devi, 2001; George et al., 2002; Milligan, 2004). Recent identification of a ligand that is able to selectively activate a heterodimer between κ-opioid and δ-opioid peptide receptor monomers and demonstration that this acts as a spinally-selective analgesic (Waldhoer et al., 2005) has significantly raised both interest and expectation in this field.

Initial studies using the HEK293 Flp-In T-REx cell system demonstrated the absolute requirement for addition of the inducing agent to allow expression of rMrgE and rMrgD receptors cloned into the Flp-In locus of these cells and that β-alanine functioned as an agonist at rMrgD but not rMrgE. Cells in which rMrgD was induced in response to treatment with doxycycline while rMrgE was expressed constitutively allowed detection of direct rMrgD/rMrgE interactions at the cell surface, whereas combinations of cells individually expressing rMrgD or rMrgE and those constitutively expressing rMrgE and harboring rMrgD at the Flp-In locus but in which expression of rMrgD protein was not induced provided important and clear-cut negative controls.

Although β-alanine caused substantial internalization of rMrgD both in cells expressing only this receptor and in those in which its expression was induced in the face of constitutive expression of rMrgE, visual inspection suggested this to be less effective in cells coexpressing rMrgE. Visual inspection of such images can provide, at best, qualitative indications. However, quantitation of the extent of β-alanine-induced internalization via cell surface ELISA confirmed significantly lower levels of rMrgD internalization in the presence of rMrgE and confirmed a lack of internalization of rMrgE whether expressed alone or in combination with rMrgD. Other studies have indicated a lack of internalization of the

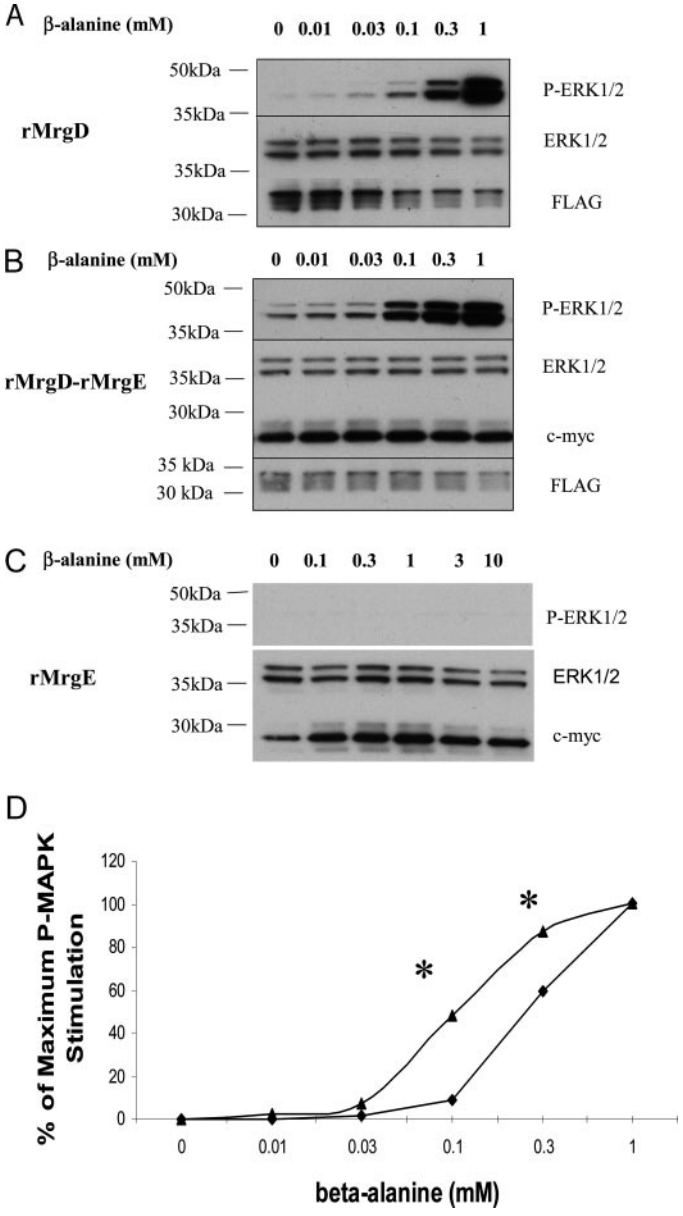


Fig. 9. Coexpression of rMrgE enhances the potency of rMrgD-β-alanine-induced ERK1/2 phosphorylation. Flp-In T-REx HEK293 cells were treated with 1 μg/ml doxycycline for 96 h to induce expression of FLAG–rMrgD in the absence (A, ♦ in D) or presence (B, ▲ in D) of constitutive expression of c-myc-rMrgE. In C, cells constitutively expressing c-myc-rMrgE were not induced. Data represent means of four independent experiments. Error bars are within the size of the symbols. *, significantly different ($p < 0.01$).

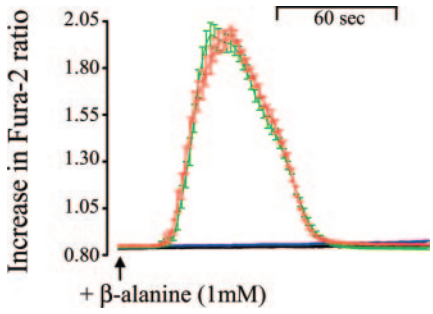


Fig. 10. Stimulation of rMrgD but not rMrgE evokes elevation of intracellular $[Ca^{2+}]$. Flp-In T-REx HEK293 cells were treated with doxycycline for 24 h to induce expression of c-myc-MrgD (red) or c-myc-MrgE (blue) receptors. As a control, cells harboring c-myc-MrgD were not induced (black). FLAG-MrgD was also induced in the presence of the constitutive expression of c-myc-MrgE (green). The effect of 1 mM β-alanine on cellular $[Ca^{2+}]_i$ was then assessed in individual cells. The data (means ± S.E.M.) are pooled from 62 cells for the noninduced samples and 43 cells in the case of each of the induced cell lines.

β_2-adrenoceptor in response to agonist ligands when coexpressed with the κ-opioid peptide receptor that is largely resistant to internalization when occupied by its own selective agonist ligands (Jordan et al., 2001). Such observations have been interpreted as an indication of heterodimerization between these two GPCRs when coexpressed (Jordan et al., 2001), although it has also been argued that although such interactions can be observed in transfected cells, this receptor pair does not form high-affinity heterodimers (Ramsay et al., 2002) and thus may be of limited importance in a physiological context. In similar studies, coexpression of the β_2-adrenoceptor with the closely related β_3-adrenoceptor has also been shown to hinder agonist-induced internalization of the β_2-adrenoceptor (Breit et al., 2004), presumably because it has been long appreciated that the β_3-adrenoceptor is internalized very poorly in response to agonists (Breit et al., 2004) and that interaction with the β_3-adrenoceptor limits internalization of a β_2-adrenoceptor-β_3-adrenoceptor heterodimer because the-β_3-adrenoceptor element is dominant in this phenotype. The internalization and β-arrestin-interaction phenotype of coexpressed GPCRs has also been examined for receptor pairs that respond to the same or similar ligands but individually display distinct β-arrestin-interaction affinities (Hanyaloglu et al., 2002; Terrillon et al., 2004). Therefore, the altered internalization characteristics of rMrgD in the presence of rMrgE are certainly compatible with their heterodimerization. The fact that a substantial fraction of rMrgD was still able to internalize in response to β-alanine in the presence of rMrgE may at first glance seem inconsistent with this model. However, it must be anticipated that when two GPCRs are coexpressed, the corresponding homodimers will also be generated, and that the proportion of homo- and heterodimers will reflect the absolute expression level of each GPCR as well as the relative propensity to form homo- and heterodimers. This is likely to be determined by their relative interaction affinity for a homomonomer and the potential heteropartner. As such, it is certainly possible that the rMrgD internalized in the presence of rMrgE is actually the fraction that represents rMrgD homodimers and that the difference in extent of internalization in the presence and absence of rMrgE expression actually represents the fraction of rMrgD present as the rMrgD-rMrgE heterodimer. These are enormously challenging questions to address directly and quantitatively, but it may be that differential two- and three-protein FRET imaging techniques with associated photobleaching protocols will be able to provide insights.

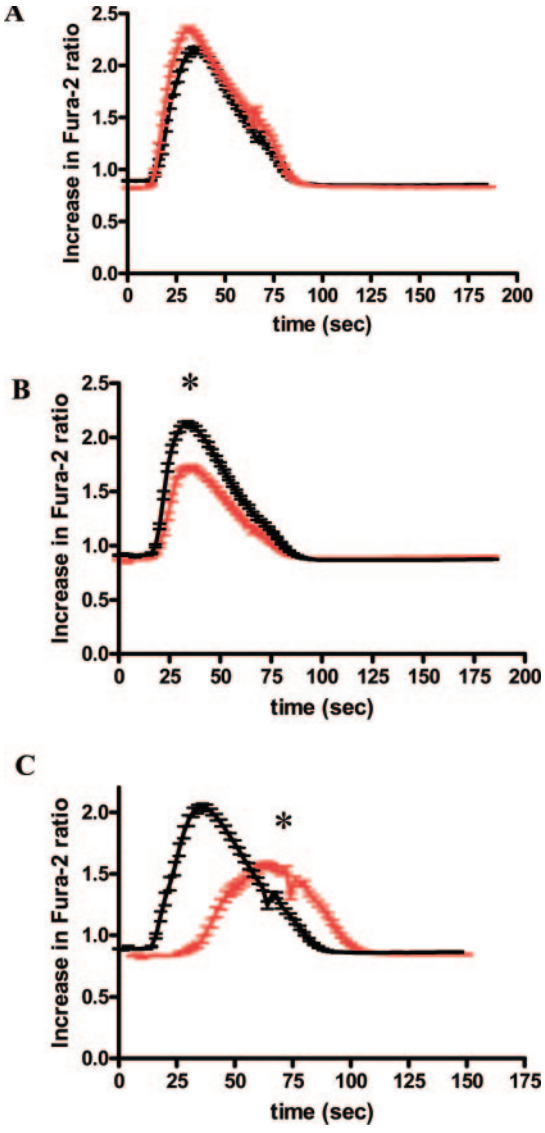


Fig. 11. Coexpression with rMrgE maintains the capacity of β-alanine to elevate intracellular [Ca^{2+}] via rMrgD. Flp-In T-REx HEK293 cells were induced to express FLAG-MrgD receptors for 24 (A), 48 (B), or 72 (C) h in the absence (red) or constitutive presence of c-myc-MrgE receptors (black). Cells were loaded with Fura-2/acetoxymethyl ester and $[Ca^{2+}]_i$ levels imaged over time after exposure to 1 mM β-alanine. Data represent means ± S.E.M from analysis of 115 cells for each condition at each time point. *, significantly different ($p < 0.001$).

To assess the functional relevance of rMrgD-rMrgE interactions, we examined two distinct signaling endpoints. β-Alanine promoted ERK MAP kinase phosphorylation via rMrgD but not rMrgE. However, in rMrgD-rMrgE coexpressing cells, although the transient nature of ERK1/2 phosphorylation was not different from cells expressing only rMrgD, there was a clear and statistically significant increase in potency of β-alanine to produce this effect. It is impossible at this stage to provide clear evidence for the mechanism responsible. The pharmacology of a number of GPCR heterodimers has been shown to be distinct from the corresponding homodimers (Maggio et al., 2005). However, because the available ligands at rMrgD are essentially restricted to β-alanine and rMrgE remains an orphan GPCR, this cannot be addressed at this point. As noted earlier, the presence of rMrgE limited the extent of β-alanine-mediated rMrgD internalization. A substantial literature has examined the importance or otherwise of receptor internalization for ERK1/2 phosphorylation and activation (Kramer and Simon, 2000; Pierce et al., 2000). However, studies with GPCRs modified to prevent internalization in response to agonist occupancy or those that are naturally resistant to agonist-induced internalization have confirmed that receptor internalization is not a prerequisite (Budd et al., 1999; Hislop et al., 2001). It is anticipated that interactions between the two elements of a GPCR heterodimer will produce allosteric effects on ligand binding (Durroux, 2005) and vice versa, and such effects may also contribute to the different potency of β-alanine observed.

One unexpected but very obvious difference in function of β-alanine in cells coexpressing rMrgD and rMrgE compared with cells expressing only rMrgD was in the regulation of

$[Ca^{2+}]_i$. Although rMrgD expression was maintained at similar levels over a period of induction of expression of between 24 and 72 h, it was obvious that both the maximal elevation of $[Ca^{2+}]_i$ was reduced at the latter time point and the kinetics of elevation were considerably slower. As this was initially surprising, we analyzed this effect in more than 100 individual cells at each time point, and thus this difference is highly significant ($p < 0.001$). However, in cells coexpressing rMrgD and rMrgE, β-alanine-mediated elevation of $[Ca^{2+}]_i$ was not different at different periods of rMrgD expression. Although the basis for this difference is unclear, these observations further indicate the importance of examining coexpressed pairs or indeed groups of receptors in concert rather than in isolation after expression.

These studies provide the first demonstration of heterodimerization between members of the Mrg family of GPCRs and highlight that many aspects of receptor function, including agonist-mediated internalization, and hence potential desensitization, and the details of agonist potency and extent of function can be altered by coexpression and heterodimerization between distinct but related GPCRs.

The current studies are limited, however, by a number of potential issues. First, because MrgE is an orphan GPCR and no high-affinity ligands of MrgD are available, it has not been possible to quantitate the expression levels of the two receptors used in these studies. It is thus unclear how these relate to expression levels within dorsal root ganglia. Second, the lack of Mrg receptor-subtype-specific antibodies limits efforts to explore direct protein-protein interactions involving MrgD and MrgE in native tissues. Finally, although the addition of a wide range of both N- and C-terminal tags frequently has little effect on the basic pharmacology and function of many GPCRs (Wilson et al., 2005), the extremely limited pharmacology currently available to explore Mrg receptor function means that we cannot state with certainly that this has not modified receptor function in the current studies. The physiological significance of in vivo oligomerization of MrgD and MrgE receptors, therefore, remains to be determined. It has been demonstrated that expression of MrgD is restricted to nociceptive neurons (Zylka et al., 2003; Shinohara et al., 2004), and this receptor is up-regulated in animal models of neuropathic pain (Shinohara et al., 2004). Based on these limited data it is interesting to speculate that the physiological interaction of these receptors in vivo could provide a level of control or fine-tuning of nociceptive processing. Future work using all available genetic and chemical tools will address these interesting and fundamental questions.

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Address correspondence to: Graeme Milligan, Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. E-mail: g.milligan@bio.gla.ac.uk