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Sensory Neuron-Specific Mas-Related Gene-X1 Receptors Resist Agonist-Promoted Endocytosis[S]

Hans Jürgen Solinski, Ingrid Boekhoff, Michel Bouvier, Thomas Gudermann, and Andreas Breit

Walther-Straub-Institut für Pharmakologie und Toxikologie, Ludwig-Maximilians-Universität München, München, Germany (H.J.S., I.B., T.G., A.B.); and Department of Biochemistry and Groupe de Recherche Universitaire sur le Médicament, Institute of Research in Immunology and Cancer, Université de Montréal, Montreal, Canada (M.B.)

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ABSTRACT

Human sensory neuron-specific mas-related gene X1 receptors (hMrgX1s) belong to the superfamily of G protein-coupled receptors (GPCRs), bind cleavage products of pro-enkephalin with high affinity, and have been suggested to participate in pain sensation. Murine or rat MrgC receptors exhibit high similarities with hMrgX1 in terms of expression pattern, sequence homology, and binding profile. Therefore, rodents have been used as an in vivo model to explore the physiological functions and pharmacodynamics of the hMrgX1. Agonist-promoted receptor endocytosis significantly affects the pharmacodynamics of a GPCR but is not yet investigated for hMrgX1. Therefore, we analyzed the effects of prolonged agonist exposure on cell surface protein levels of hMrgX1 and murine or rat MrgC in human embryonic kidney 293, Cos, F11, and ND-C cells. We observed that hMrgX1 are resistant and both MrgC are prone to agonist-promoted receptor endocytosis. In Cos cells, coexpression of β-arrestins strongly enhanced endocytosis of murine MrgC but did not alter cell surface expression of hMrgX1 receptors. These data define the hMrgX1 as one of the few members within the superfamily of GPCRs whose signaling is not regulated by agonist-promoted endocytosis and reveal species-specific differences in the regulation of Mrg receptor signaling. Given the importance of receptor endocytosis for the pharmacodynamics of a given ligand, our results may have a strong impact on the development of future drugs that suppose to control pain in humans but were tested in rodents.

The family of human mas-related gene X receptors (hMrgXs) comprises four receptor subtypes that belong to the superfamily of G protein-coupled receptors (GPCRs) (Dong et al., 2001). Of these four subtypes, only the hMrgX1 and -2 are further characterized. hMrgX1s are exclusively expressed in dorsal root ganglia (DRG) neurons (Dong et al., 2001; Lembo et al., 2002), whereas hMrgX2s are additionally expressed in many other tissues (Robas et al., 2003). The endogenous ligands of hMrgX1 originate from pro-enkephalin that also gives rise to opioids such as leu- and met-enkephalin (Lembo et al., 2002). In addition, pro-enkephalin produces biologically active compounds named bovine adrenal medulla (BAM) peptides that have been found to occur under physiological conditions (Höllt et al., 1982; Dores et al., 1990). BAM22 has the ability to activate hMrgX1 and opioid receptors, because its extreme N terminus harbors the classic enkephalin motif that is responsible for high-affinity binding to opioid receptors, whereas the C-terminal portion of BAM22 binds hMrgX1. Full agonistic activity toward hMrgX1 is preserved after the removal of seven N-terminal residues, because the BAM8–22 fragment exhibits similar high potency and efficacy to activate Mrg receptors compared with the entire BAM22 peptide (Lembo et al., 2002; Grazzini et al., 2004). Among rodents, no clear orthologs of hMrgX1 could be found (Burstein et al., 2006). However, murine and rat MrgC receptors share ~65% sequence homology and similarities in terms of expression pattern and binding profile with hMrgX1 (Dong et al., 2001; Han et al., 2002; Lembo et al., 2002; Grazzini et al., 2004). Therefore, rodents seem reasonable model systems to investigate the physiological role of BAM8–22 and

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ABBREVIATIONS: hMrgX1, human sensory neuron-specific mas-related gene X1 receptor; AR, adrenergic receptor; β-arr, β-arrestin; AUC, area under the curve; BAM, bovine adrenal medulla; BSA, bovine serum albumin; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; Ex, Xpress epitope; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HBS, HEPES-buffered saline; HEK, human embryonic kidney; Mrg, mas-related gene; MCR, melanocortin receptor; MSH, melanocyte-stimulating hormone; PBS, phosphate-buffered saline; SNSR, sensory neuron-specific G protein-coupled receptor; YFP, yellow fluorescent protein.

hMrgX1. In addition to BAM peptides, the pro-opiomelanocortin cleavage product γ_2-MSH has also been shown to activate Mrg receptors. For mMrgC receptors, BAM8–22 and γ_2-MSH were found to be equally potent (Han et al., 2002), whereas in hMrgX1-expressing cells, BAM8–22 (Lembo et al., 2002), and in rMrgC-expressing cells (Grazzini et al., 2004), γ_2-MSH seemed to be more potent than the other. Thus, although hMrgX1 and rodent MrgC have many attributes in common, they also show slight differences in their ligand binding profile.

Given the exclusive expression of hMrgX and MrgC in DRG, it has been proposed that they are involved in controlling nociception and therefore represent promising therapeutic targets for pain therapy. In agreement with this prediction, the application of BAM8–22 increased pain-averting behavior in rats or mice after challenging animals with heat (Grazzini et al., 2004; Chang et al., 2009), pointing to algetic actions of this peptide. However, since then, conflicting data have been reported, because BAM8–22 has also been shown to decrease nociception in rats after challenging animals with either formalin or heat, thus also pointing to an analgetic role of BAM8–22-promoted signaling (Hong et al., 2004; Zeng et al., 2004; Chen et al., 2006).

Considering the G protein-coupling properties of Mrg receptors, all studies agree that they activate phospholipases C via G_q proteins (Dong et al., 2001; Han et al., 2002; Lembo et al., 2002; Breit et al., 2006a), whereas some studies suggest the involvement of pertussis toxin-sensitive G proteins in hMrgX1-induced signaling (Chen and Ikeda, 2004; Galés et al., 2005; Burstein et al., 2006). The pain-mediating vanilloid receptor-1 (Honan and McNaughton, 2007; Hager et al., 2008), voltage-gated calcium channels, and M-type potassium channels (Chen and Ikeda, 2004) are the only known downstream effectors of Mrg receptor signaling. In addition to the incomplete picture of Mrg receptor-induced signaling to date, no data are available regarding the agonist-promoted regulation of hMrgX1 signaling.

To analyze the regulatory processes controlling Mrg receptor signaling, we created HEK293 cell lines stably expressing either the mMrgC or the hMrgX1 receptor. Monitoring agonist-promoted calcium transients in cells pretreated with BAM8–22, we were surprised to observe significant agonist-promoted desensitization in cells expressing mMrgC- but not in hMrgX1-expressing cells. Furthermore, although the mMrgC subtype underwent robust agonist-promoted receptor endocytosis, its human counterpart was resistant to this regulatory process. In Cos cells that have been shown to express low levels of β-arrestins (Ménard et al., 1997; Zhang et al., 1997), significant mMrgC endocytosis was not detectable but could be retrieved by recombinant coexpression of either β-arrestin-1 or -2, suggesting that mMrgC but not hMrgX1 functionally interacts with arrestins. Differences in agonist-promoted receptor endocytosis among distinct species were also detectable in a more physiologically relevant cellular setting because they were found in two DRG-like cell lines recombinantly expressing either receptor. It is noteworthy that the rat MrgC was also found to be sensitive to receptor endocytosis in the same settings, strongly indicating that regulation of human and rodent BAM8–22-sensitive Mrg receptors is fundamentally different.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium, Ham's F-12 nutrient mixture, fetal bovine serum (FBS), penicillin/streptomycin, phosphate-buffered saline (PBS), trypsin/EDTA, Zeocin, G418, and hypoxanthine/amitriptyline/thymidine supplement were purchased from Invitrogen (Carlsbad, CA). Metafectene was obtained from Biontex (Munich, Germany), and PromoFectin was from PromoCell (Heidelberg, Germany). Murine Anti-Xpress antiserum was obtained from Invitrogen, and horseradish peroxidase-conjugated anti-mouse antibody, raised in goat, was obtained from Sigma-Aldrich (Deisenhofen, Germany). 3,3′,5,5′-Tetramethylbenzidine ELISA substrate was from Thermo Fisher Scientific (Waltham, MA), *o*-phenylenediamine ELISA-substrate, BSA, pluronic F-127, and poly(L-lysine) were from Sigma-Aldrich, and fura-2-acetoxymethyl ester was obtained from Fluka (Deisenhofen, Germany). BAM22 and BAM8–22 were purchased from Biotrend (Cologne, Germany). Carbachol and α- and γ_2-MSH were from Sigma-Aldrich.

Eukaryotic Expression Vectors. For endocytosis experiments, expression vectors encoding the human hMrgX1 (GenBank accession number AF474990), the rat rMrgC (GenBank accession number AF518245), or the murine mMrgC (GenBank accession number AY152435) fused to the Xpress epitope (pcDNA4-hMrgX1, pcDNA4-rMrgC, and pcDNA4-mMrgC) were generated as follows: polymerase chain reaction fragments containing the entire coding sequences excluding the start codon of the human hMrgX1 (forward primer, 5′-ATCGATCCAACGGTCTCAACC-3′; reverse primer, 5′-CGTCTAGATCACTGCTCC-AATCTGC-3′), the rat rMrgC (forward primer, 5′-ATCGATCCAACCATCTCATCC-3′; reverse primer, 5′-CGTCTAGATCAACATCTCCTTTCTG-3′), or the murine mMrgC (forward primer, 5′-ATC-GATCCAACCATCTCATCC-3′; reverse primer, 5′-CGTCTAGATCAATATCTGCTTTCTG-3′) were subcloned into the pcDNA4 vector (Invitrogen) in a way that fused the 5′-end of the receptors to the 3′-end of the Xpress epitope using the restriction sites EcoRV and XbaI, respectively. The integrity of these new constructs was verified by DNA sequence analysis. The construction of the Ex-hMC4R and Ex-hMC3R plasmids (Breit et al., 2006b) and of the β-arrestin-1-YFP and β-arrestin-2-YFP plasmids (Hoffmann et al., 2008) has been reported previously.

Cell Culture and Transfection. HEK293, Cos, and ND-C cells (obtained from the Health Protection Agency Culture Collection, Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). F11 cells were cultured in Ham's F-12 nutrient mixture supplemented with 20% FBS, 2 mM L-glutamine, hypoxanthine/amitriptyline/thymidine supplement, penicillin (100 U/ml), and streptomycin (100 μg/ml). For transient expression, cells were seeded at a density of 2×10^6 cells in a 10-cm dish, cultured for 24 h, and then transfected with the appropriate vectors using Metafectene reagent (HEK293 cells) or PromoFectin reagent (Cos, F11, and ND-C cells), according to the manufacturer's protocol. HEK293 cell clones stably expressing human hMrgX1 (HEK293-Ex-hMrgX1 cells), rat rMrgC (HEK293-Ex-rMrgC cells), or murine mMrgC (HEK293-Ex-mMrgC cells) were obtained by selecting cells (400 μg/ml Zeocin; Invitrogen) transfected with the pcDNA4-hMrgX1, pcDNA4-rMrgC, or pcDNA4-mMrgC construct, respectively. F11 cell clones stably expressing human hMrgX1 (F11-Ex-hMrgX1 cells) were obtained by selecting cells (250 μg/ml G418) cotransfected with the pcDNA4-hMrgX1 and empty pcDNA3.1. The expression of the fusion protein was controlled by measuring agonist-induced calcium release and by measuring the presence of Xpress epitopes at the plasma membrane.

Determination of Intracellular Calcium Transients. Twenty-four hours before the measurement, 2 to 3×10^6 cells were seeded in a 10-cm dish and then loaded with 5 μM (HEK293) or 10 μM (F11) fura-2-acetoxymethyl ester in HBS buffer (10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 140 mM NaCl, 0.1% glucose, and 2 mM $CaCl_2$ adjusted to pH 7.4 with 1 M NaOH) for 30 min at 37°C. In the case

of F11 cells, labeling of cells was improved by adding 0.02% Pluronic F-127 to the labeling buffer. After harvesting the cells in HBS, ~100,000 cells per well were seeded in 96-well plates, and fluorescence was measured in a FLUOstar Omega plate reader (BMG, Offenburg, Germany) at 37°C. HBS as a control or HBS including the corresponding ligand was automatically injected 5 to 10 s after starting the measurement. In intervals of 1.14 s, total emission (520 ± 20 nm) was measured after excitation of the sample with 340 ± 15 or 380 ± 15 nm. Fura-2-ratios (340:380) were then plotted against the time in seconds. To quantify ligand-promoted calcium signals of pretreated and control cells, the area under the curve (AUC) between the time points of 10 and 40 s was determined. AUC of control cells was defined as 100%.

Cell Surface ELISA. Xpress epitope fusion protein-expressing cells were detached and seeded in 12-well dishes (~200,000 cells/well) coated with 0.1% poly(L-lysine). After 24 h, Xpress epitope receptor fusion proteins were stimulated with the indicated concentrations of the corresponding ligand diluted in the appropriate serum-free medium for different times at 37°C. After stimulation, cells were immediately cooled on ice to impede possible recycling events. The ligand was thoroughly washed away with ice-cold PBS/0.5% BSA, and then Xpress epitope receptor fusion proteins were detected on the cell surface by incubating the cells with 1.1 μg/ml anti-Xpress antibody in PBS/0.5% BSA for 60 min at 4°C. Thereafter, cells were fixed on ice for 10 min with 4% paraformaldehyde and 100 mM $NaPO_4$. After washing the cells once with PBS/0.5% BSA, cells were incubated for 60 min with anti-mouse horseradish peroxidase-conjugated secondary antibodies from goat (1:3000) in PBS/0.5% BSA at room temperature. Then, cells were washed twice for 20 min with PBS/0.5% BSA and twice with PBS. 3,3′,5,5′-tetramethylbenzidine or *o*-phenylenediamine ELISA-substrate was added according to the manufacturer's instructions, and extinction was measured at 450 or 492 nm, respectively.

YFP Measurement. To control for the expression of β-arrestin-1-YFP or β-arrestin-2-YFP fusion proteins, ~200,000 Cos cells, derived from the same transfection used for the endocytosis experiment, were seeded in six-well dishes 24 h after transfection. One day later, cells were detached and seeded onto 96 wells, and YFP fluorescence (excitation filter, 480 ± 15 nm; emission filter, 535 ± 10 nm) was determined with a FLUOstar Omega plate reader. Background values of mock-transfected cells were subtracted.

Data Analysis. Data obtained by cell surface ELISA, YFP, and calcium measurements were analyzed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Statistical significance of the differences was assessed by the two-tailed Student's *t* test.

Results

Calcium Signaling and Receptor Endocytosis in HEK293 Cells Stably Expressing Mrg Receptors. To establish a reliable cell model that stably expresses either the human MrgX1 (hMrgX1) or the mouse MrgC (mMrgC), we transfected the corresponding cDNA encoding each receptor fused with the sequence of the Xpress (Ex) epitope to its 5′-end into HEK293 cells and treated these cells with Zeocin for 3 weeks. Performing ELISA experiments, we determined cell surface expression levels of Ex-Mrg fusion proteins. As shown in Fig. 1A, both cell pools exhibited significantly increased immune reactivity compared with control cells, indicating that both receptor variants, Ex-hMrgX1 and Ex-mMrgC, are expressed at the surface of HEK293 cells. However, receptor expression levels were approximately three times higher in HEK293-Ex-hMrgX1 compared with HEK293-Ex-mMrgC cells. The BAM8–22 peptide has been shown to selectively bind and activate both hMrgX1 and mMrgC (Han et al., 2002; Lembo et al., 2002). In agreement with these previous observations, BAM8–22 induced robust calcium transients in HEK293 cells stably expressing either receptor, whereas mock-transfected cells were unresponsive (Fig. 1B). In addition to BAM peptides, γ_2-MSH has also been described to activate mMrgC (Han et al., 2002) and hMrgX1 (Lembo et al., 2002) receptors. These studies revealed that γ_2-MSH was less potent than BAM8–22 in hMrgX1-expressing cells but equally potent in mMrgC cells. However, in our cells and settings, we obtained significant signals with 1 μM γ_2-MSH in mMrgC but not in hMrgX1-expressing cells (Fig. 1C). Thus, for the further analysis of the regulation of agonist-promoted Mrg receptor signaling, we used BAM peptides for the hMrgX1 and γ_2-MSH for the mMrgC.

To get a first idea about the regulation of Mrg receptor signaling, we analyzed the effects of sustained agonist expo-

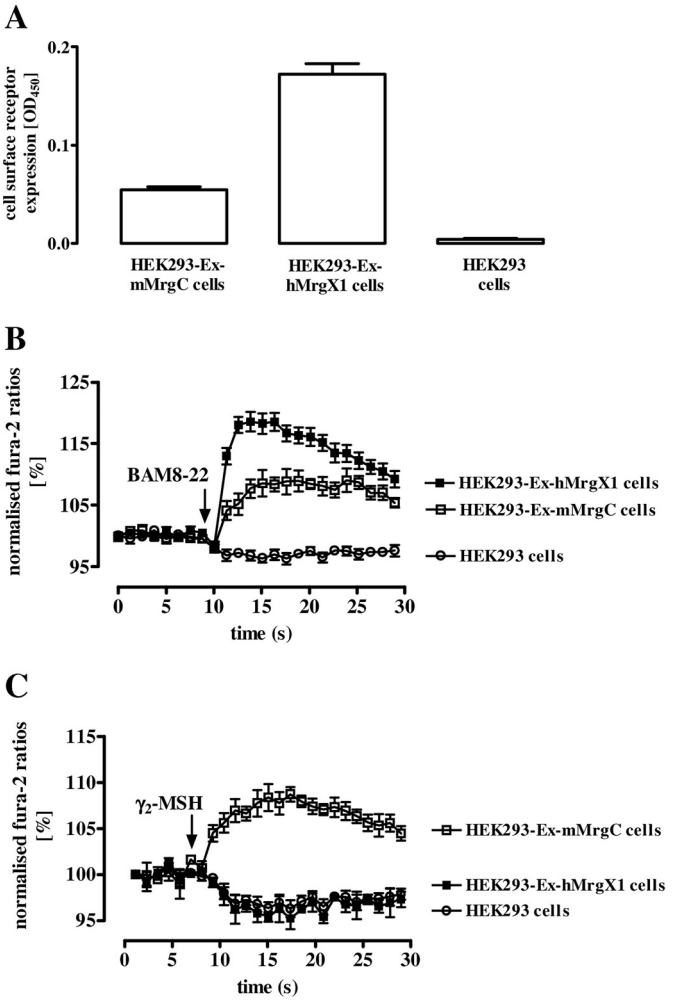

Fig. 1. Stable expression of mouse MrgC or human MrgX1 in HEK293 cells A, receptor cell surface expression in HEK293 cells stably expressing the mMrgC or the hMrgX1 fused on the N terminus with the Xpress epitope was monitored by the ELISA technique with intact cells. As a negative control, background values of mock-transfected HEK293 cells were determined. Results are expressed as the mean ± S.E. of three independent experiments performed in quadruplicates. B and C, calcium signals in fura-2-loaded HEK293-Ex-hMrgX1, HEK293-Ex-mMrgC, or mock cells were measured after injection of BAM8–22 (2 μM, final concentration in B) or γ_2-MSH (2 μM, final concentration in C) at time point 10 s. As a negative control, only HBS buffer has been injected. Data have been normalized by defining the first ratio (1.14 s) measured as 100%. Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate.

sure on Mrg receptor-induced calcium transients. Therefore, we prestimulated fura-2-labeled cells with 1 μM BAM8–22 for 30 min at 37°C and compared their abilities to yield BAM8–22-promoted calcium transients with those of untreated control cells. As expected from a given GPCR, sustained prestimulation of HEK293-Ex-mMrgC cells reduced the ability of BAM8–22 to promote calcium signals. In fact, the AUC of BAM8–22-induced calcium signals were reduced by 60% compared with control cells (Fig. 2A). Prestimulation of the same cells had no overall detrimental effects on the ability of HEK293 cells to respond with calcium signals, because agonist stimulation of the endogenously expressed muscarinic-3 receptor (Luo et al., 2008) was unaffected (Fig. 2B). We were surprised to find that BAM8–22 preincubation of HEK293-Ex-hMrgX1 cells did not affect the ability of hMrgX1 to induce calcium signals at all (Fig. 2C),

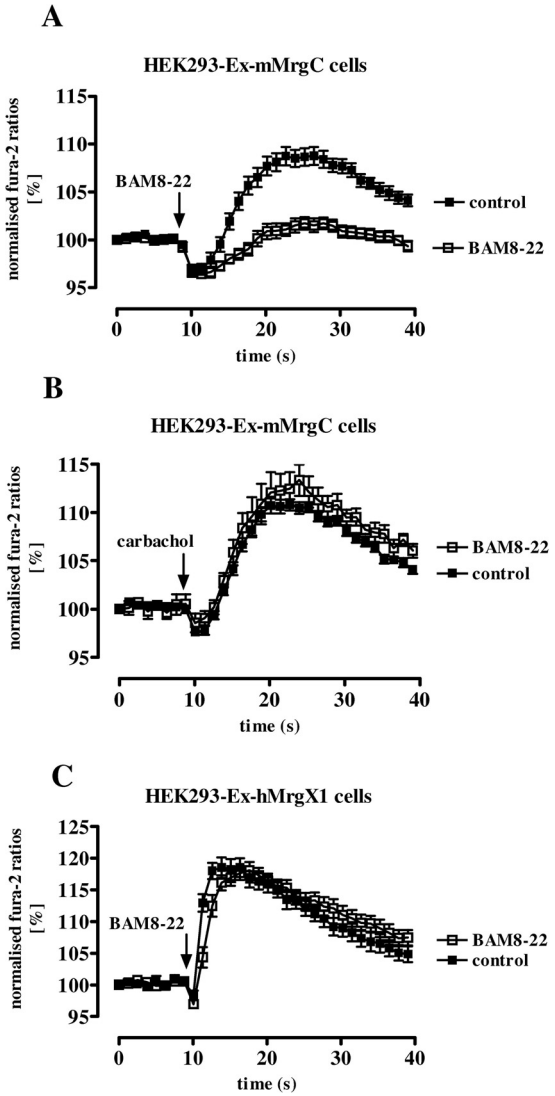

Fig. 2. Agonist-promoted desensitization of Mrg receptor-induced calcium signaling in HEK293 cells after stable protein expression. Calcium signals in control or with BAM8–22 (1 μM) pretreated (30 min at 37°C) fura-2-loaded HEK293-Ex-mMrgC (A and B) or HEK293-Ex-hMrgX1 cells (C) were measured after injection of BAM8–22 (A and C; final concentration, 2 μM) or carbachol (B; final concentration, 100 μM) at time point 10 s. Data have been normalized by defining the first ratio (1.14 s) measured as 100%. Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate.

suggesting species-specific differences in the regulation of Mrg receptor calcium signaling. To evaluate a possible molecular mechanism responsible for these differences, we analyzed the effects of sustained agonist exposure on cell surface receptor levels by ELISA experiments with intact cells. In line with the lack of agonist-induced desensitization, stimulation of HEK293-Ex-hMrgX1 cells with either 1 or even 5 μM BAM8–22 for 30 min at 37°C did not reduce the amount of receptor fusion proteins accessible to the antibody (Fig. 3, A and B), suggesting that hMrgX1-induced calcium signals are not regulated by agonist-promoted endocytosis. In line with this notion, the extended peptide BAM22, which has been described to be a bivalent agonist for Mrg and opioid receptors (Lembo et al., 2002; Breit et al., 2006a), also failed to induce hMrgX1 endocytosis (Fig. 3, A and B). In contrast, HEK293-Ex-mMrgC cells showed a profound loss of mMrgC receptor proteins at the cell surface after treatment with either BAM8–22 or γ_2-MSH (Fig. 3, A and B). The extent and kinetics of mMrgC endocytosis were comparable with endocytosis expected from a GPCR (Fig. 3C), suggesting that in contrast to hMrgX1, mMrgC signaling is regulated by agonist-promoted endocytosis.

Agonist-promoted Mrg receptor endocytosis in HEK293 cells after transient expression. It is noteworthy that the receptor expression level, which could negatively influence receptor endocytosis, is approximately three times higher in HEK293-Ex-hMrgX1 compared with HEK293-Ex-mMrgC cells. To exclude the effects of receptor expression levels on Mrg receptor endocytosis, we transiently expressed Ex-hMrgX1 or Ex-mMrgC proteins in HEK293 cells. As shown in Supplementary Figure 1, A and B, receptor expression levels between optical density readings of 0.02 to 0.25 were achieved. Within this range, agonist-promoted endocytosis could be detected for mMrgC but not for the hMrgX1, suggesting that discrepancies between receptor expression levels do not account for the differences in endocytosis. Because the extent of ligand-promoted Ex-mMrgC endocytosis observed for BAM8–22 or γ_2-MSH was rather low (~20 and ~10%, respectively), we compared these values with those obtained with the human MC3R or MC4R (Supplementary Fig. 1C), which have been established recently as GPCR that undergo robust endocytosis in HEK293 cells (Shinyama et al., 2003; Breit et al., 2006b; Wachira et al., 2007). As summarized in Supplementary Fig. 1D, MCR endocytosis induced by α-MSH, reached ~15% for either MCR, and therefore matched the results obtained with mMrgC.

Agonist-Promoted Mrg Receptor Endocytosis in F11 Cells. Regulation of receptor signaling might be different depending on the cellular context. In particular, the lack of agonist-promoted desensitization of hMrgX1 might be an HEK293 cell-specific artifact. However, it is important to note that neither mMrgC nor MCR is endogenously expressed in HEK293 cells but exhibit an endocytosis profile as expected from a given GPCR, indicating that, in principle, HEK293 cells are suitable to detect GPCR endocytosis. Nevertheless, to exclude that differences in the behavior of hMrgX1 and mMrgC are restricted to HEK293 cells, we took advantage of F11 cells that represent a DRG-like cell line (hybridoma of mouse neuroblastoma × rat DRG cells) and endogenously express proteins usually found in DRG such as the bradykinin-2 receptor (Platika et al., 1985). As shown in Fig. 4A, F11 cells strongly responded to bradykinin, but no calcium signals were obtained with Mrg receptor-specific agonists, indicating that F11 cells do not

endogenously express Mrg receptors. Thus, we transiently overexpressed hMrgX1 or mMrgC receptors in F11 cells. In addition, to obtain a more detailed picture of the regulation of Mrg receptor signaling among rodents, we included the rat MrgC (rMrgC) in our study. Measuring agonist-induced calcium transients in F11 cells expressing hMrgX1, mMrgC, or rMrgC, we observed that BAM8–22 is a full agonist for all three Mrg receptors (Fig. 4). γ_2-MSH appeared as a strong partial agonist (~92%) of the mMrgC (Fig. 4C) and, similar to our results obtained in HEK293 cells (Fig. 1C), did not activate the hMrgX1 (Fig. 4B). In the case of the rMrgC, γ_2-MSH and BAM8–22 were equally efficient (Fig. 4D). To assess receptor endocytosis, we stimulated Mrg receptor-expressing F11 cells with the corresponding peptides for 30 min at 37°C. Thereby, we detected a robust reduction of Ex-mMrgC but not of Ex-hMrgX1 proteins on the cell surface (Fig. 5A, B, and D) and thus confirmed our results obtained with HEK293 cells. It is noteworthy that stimulation of rMrgC-expressing F11 cells with equipotent concentrations of γ_2-MSH (1 μM) or BAM8–22 (5 μM) also revealed a loss of receptor numbers on the cell surface (Fig. 5, C and D), indicating that rMrgC, similar to mMrgC, undergo agonist-promoted receptor endocytosis in F11 cells. To substantiate this finding, we also created an HEK293 cell line stably expressing the Ex-rMrgC protein (HEK293-Ex-rMrgC cells). Measurements of intracellular calcium transients in these cells revealed a similar pattern of cellular responses after γ_2-MSH and BAM8–22 challenge (Supplementary Fig. 2A) compared with F11 cells (Fig. 4D). Fur-

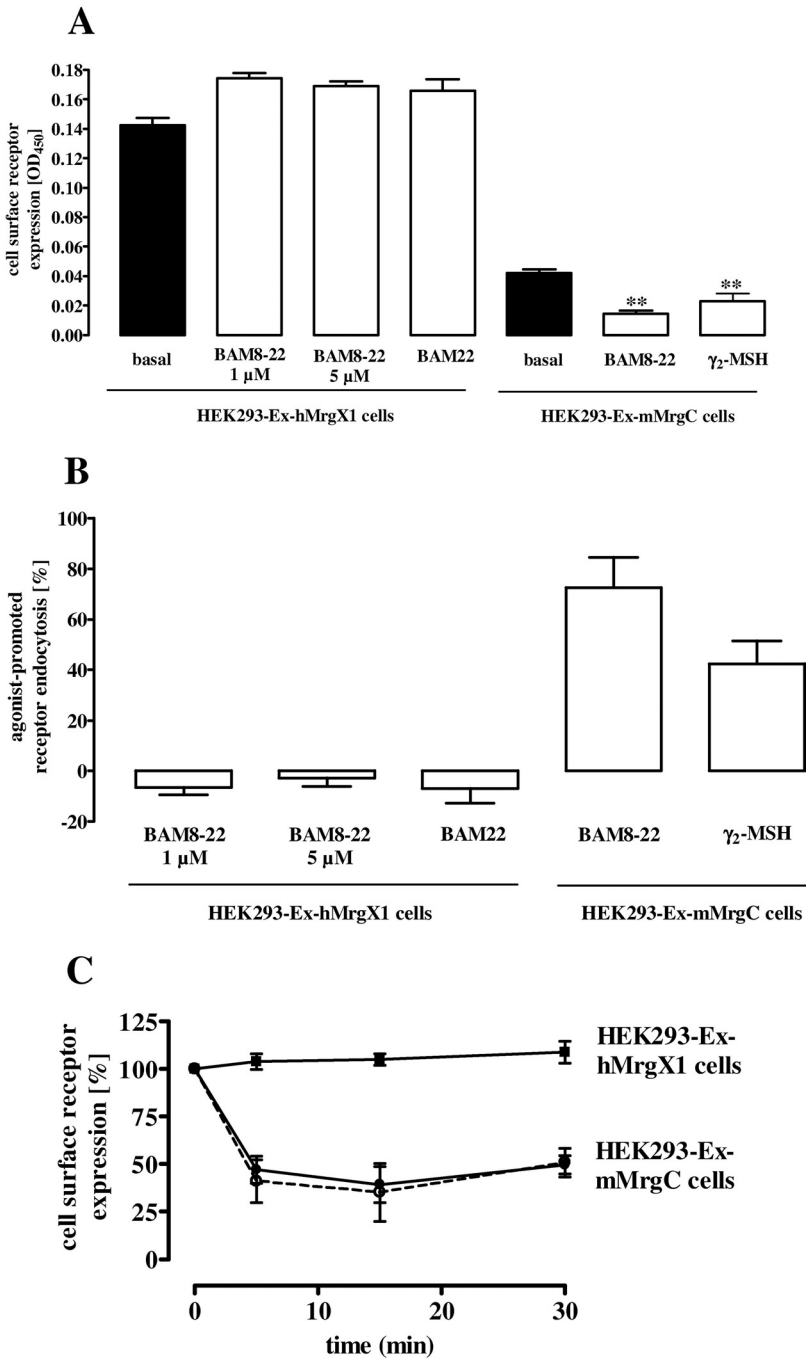

Fig. 3. Agonist-promoted endocytosis of Mrg receptors in HEK293 cells after stable protein expression. Receptor cell surface expression of control or with BAM8–22 (1 or 5 μM) or BAM22 (1 μM) treated (30 min at 37°C) HEK293-Ex-hMrgX1 or with BAM8–22 (1 μM) or γ_2-MSH (1 μM) treated HEK293-Ex-mMrgC cells was monitored by the ELISA technique with intact cells. A, absolute values of one representative experiment carried out in quadruplicate are shown. Background values of mock-transfected cells have been subtracted. Results are expressed as the mean ± S.E. Asterisks indicate a significant (**, $p < 0.01$) difference between treated and untreated cells. B, data of five independent experiments are presented as ligand-promoted endocytosis in percentage. C, HEK293-Ex-hMrgX1 cells were stimulated with 1 μM BAM8–22 (■) or HEK293-Ex-mMrgC cells with 1 μM BAM8–22 (●) or γ_2-MSH (○) for 5, 15, or 30 min at 37°C. Receptor cell surface expression is given as percentage (with time point 0 defined as 100%) and plotted against time of stimulation. Results are expressed as the mean ± S.E. of three independent experiments carried out in triplicate.

thermore, both peptides were able to induce significant receptor endocytosis in these cells (Supplementary Fig. 2, B and C). Thus, we conclude that similar to its murine ortholog, signaling induced by rMrgC is regulated by agonist-promoted receptor endocytosis in HEK293 cells.

Next, we sought to establish F11 cell lines that stably express either Mrg receptor protein. Although, we were able to obtain F11 cells stably expressing Ex-hMrgX1, no cells were found expressing mMrgC or rMrgC. However, as shown in Fig. 6A cell surface expression of hMrgX1 was rather low in these cells. It is noteworthy that despite these low expression levels, marked calcium signals could be evoked (Fig. 6B), which clearly exceeded those obtained in HEK293-Ex-hMrgX1 cells (Fig. 1B or 2C). This favorable ratio of calcium signal over cell surface expression might reflect very efficient coupling and signaling of hMrgX1 in F11 cells. Regardless of the efficient coupling and low expression level, no ligand-induced endocytosis of hMrgX1 was detectable in these cells (Fig. 6A). Thus, it seems that resistance of hMrgX1 to agonist-promoted endocytosis is not restricted to HEK293 cells but also occurs in a DRG-like cell line.

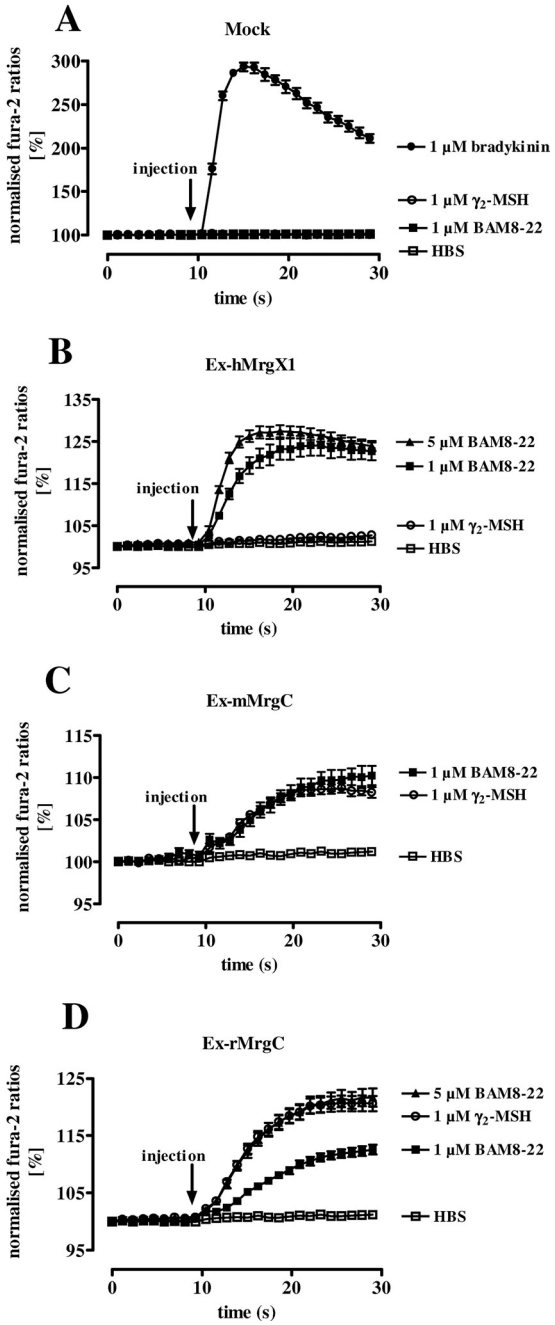

Fig. 4. Calcium signals in F11 cells transiently expressing Mrg receptors. Calcium signals in fura-2-loaded naive F11 cells (A) or cells transiently expressing either Ex-hMrgX1 (B), Ex-mMrgC (C), or Ex-rMrgC (D) after injection of BAM8–22 or γ_2-MSH (final concentrations as indicated) at time point 10 s were measured. A, bradykinin served as a positive control; A to D, only HBS buffer was injected as a negative control. Data were normalized by defining the first ratio (1.14 s) measured as 100%. Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate.

Agonist-Promoted Mrg Receptor Endocytosis in ND-C Cells. Given the exclusive expression pattern of all three Mrg receptors in DRG, we tested species-specific alterations of receptor endocytosis in a second DRG-like cell line. ND-C cells have been established as a model system to analyze the pharmacological properties of DRG-specific proteins on the cellular level (Wood et al., 1990; Tang et al., 1994; Wu et al., 2008); thus, we have chosen these cells to further determine species-specific differences in Mrg receptor endocytosis. Stimulation of ND-C cells transfected with the respective cDNAs coding for Ex-tagged Mrg receptors confirmed that both rodent MrgC receptors are prone to agonist-promoted endocytosis either induced by BAM8–22 or γ_2-MSH and that hMrgX1s resist BAM8–22-promoted endocytosis (Fig. 7, A–D).

***β*-Arrestin-Dependent Agonist-Promoted mMrgC Endocytosis in Cos Cells.** Members of the arrestin family (*β*-arr-1 and *β*-arr-2) play an important role in initiating and executing GPCR endocytosis (Ferguson et al., 1996). It has been reported that GPCRs (e.g., the β_2-adrenergic receptor) internalize poorly in Cos cells that express low levels of endogenous arrestins (Ménard et al., 1997; Zhang et al., 1997). Therefore, the Cos cell model represents an excellent tool to analyze the dependence of GPCR endocytosis on the functional interactions with arrestins. To obtain first data about putative interactions of Mrg receptors with arrestins, we transiently expressed the hMrgX1 and the mMrgC with or without arrestins in Cos cells and determined agonist-promoted endocytosis in these cells. In agreement with our results obtained in all cell models tested previously, hMrgX1 endocytosis was also absent in Cos cells and unaffected from overexpression of either *β*-arr-1 or *β*-arr-2 (Fig. 8), suggesting that this receptor subtype does not functionally interact with arrestins. On the other hand, in mMrgC expressing Cos cells, coexpression of *β*-arr-1 or *β*-arr-2 dramatically enhanced agonist-promoted receptor endocytosis (Fig. 8), indicating that functional interactions between arrestins and mMrgC receptors are required for agonist-promoted mMrgC endocytosis and that differential endocytosis among Mrg receptors may reflect different affinities of these receptors to arrestins.

Discussion

The present study was undertaken to study agonist-promoted endocytosis of BAM8–22-sensitive Mrg receptors from different species. Performing experiments in HEK293, Cos, F11, and ND-C cells, we conclude that the human MrgX1 is

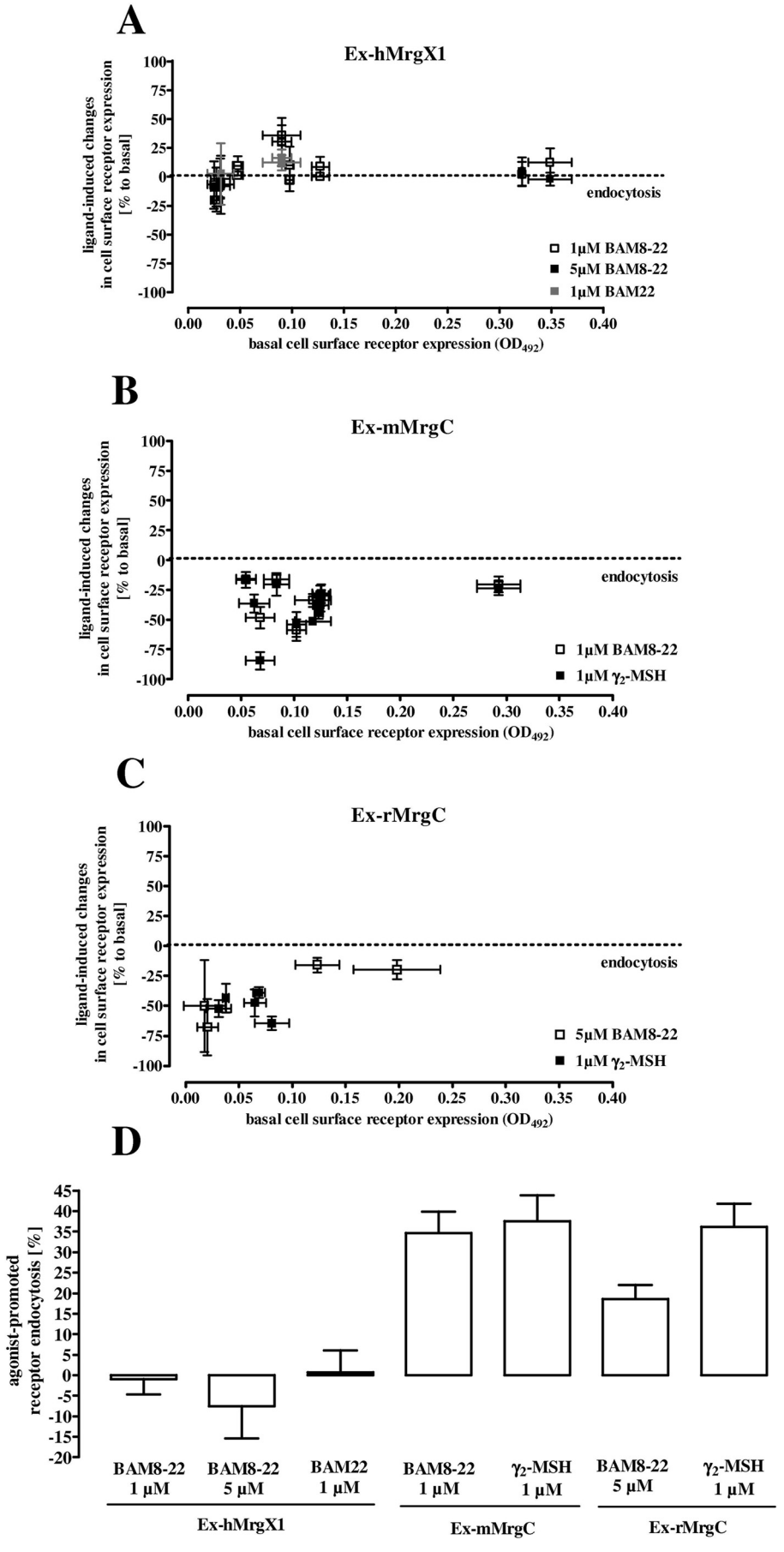

Fig. 5. Agonist-promoted endocytosis of Mrg receptors in F11 cells. A to C, cell surface receptor expression under basal conditions (*x*-axis) monitored by the ELISA technique with intact cells is plotted against ligand-induced changes in cell surface receptor expression (*y*-axis). A, Ex-hMrgX1-expressing cells were stimulated with 1 μM BAM8–22 (□) or 5 μM (■) or 1 μM BAM22 (▨). B, Ex-mMrgC-expressing cells were stimulated with 1 μM BAM8–22 (□) or 1 μM γ_2-MSH (■). C), Ex-rMrgC-expressing cells were stimulated with 5 μM BAM8–22 (□) or 1 μM γ_2-MSH (■). Incubations were carried out for 30 min at 37°C. Background values of mock-transfected cells have been subtracted. D, the same data are presented as ligand-promoted endocytosis as percentages. Results are expressed as the mean ± S.E. of 4 to 10 independent experiments carried out in quadruplicate.

one of the few members within the superfamily of GPCR whose signaling is resistant to agonist-promoted endocytosis. Furthermore, we observed species-specific differences in the response of Mrg receptors to sustained agonist exposure.

Our unexpected findings in the regulation of rodent MrgC and hMrgX1 raised the question about the mechanisms responsible for the differences observed. Because the same peptide solution was used for each receptor in each experiment, we exclude that artificial inactivation of the peptides accounts for absent endocytosis. Furthermore, we exclude clonal variability as a possible explanation, because it was observed in a second, independent HEK293 cell clone (data not shown) and in F11 cells stably expressing hMrgX1. Increased receptor expression levels have been shown to negatively affect receptor endocytosis (Breit et al., 2006b), most probably because of unfavorable expression ratios of the receptor protein and proteins required for receptor endocytosis (e.g., arrestins). Considering higher receptor levels in HEK293-Ex-hMrgX1 cells compared with HEK293-Ex-mMrgC cells, this might be of particular interest. However, differences in MrgC and hMrgX1 endocytosis also prevailed after transient protein expression, although MrgC expression levels were similar to or even higher than those of the hMrgX1, indicating that increased hMrgX1 expression levels do not account for the observed differences. Distinctions in endocytosis observed among Mrg receptors are also not due to distinct signaling profiles, because in HEK293 and F11 cells, similar agonist-promoted calcium signaling was observed. Given the higher calcium signals observed in hMrgX1- compared with MrgC-expressing cells, we assume that neither the quality nor the quantity of Mrg receptor signaling is responsible for the differences in receptor endocytosis. Thus, it seems that at least in HEK293, F11, and ND-C cells, hMrgX1s resist agonist-promoted receptor endocytosis under conditions that allow for robust MrgC endocytosis. Cytosolic adapter proteins of the arrestin family (e.g., β-arr-1 or -2) are critical components of the cellular machinery that controls and executes GPCR endocytosis into clathrin-coated pits (Ferguson et al., 1996). In contrast, endocytosis of cell surface proteins into caveolae (smaller vesicles containing the cholesterol-binding protein caveolin) is independent of arrestins. It has been proposed that Cos cells express low levels of endogenous arrestins (Ménard et al., 1997; Zhang et al., 1997) and thus represent a useful tool to discriminate between receptor endocytosis into clathrin-coated pits and caveolae. Using Cos cells recombinantly overexpressing β-arr-1 or -2 with either the hMrgX1 or the mMrgC, we can clearly show that mMrgC but not hMrgX1 receptors undergo agonist-promoted endocytosis in an arrestin-dependent manner, suggesting that differential endocytosis among Mrg receptors is reflected by different affinities to arrestins. It is noteworthy that arrestins not only terminate GPCR signaling but also initiate G-protein-independent signaling on their own (Shenoy and Lefkowitz, 2005). Thus, suspected differences in the interactions of arrestins with Mrg receptors of different species might not only affect the kinetics of Mrg receptor-induced signaling but could also have qualitative effects on Mrg receptor signaling.

However, it is not yet confirmed whether our findings reflect the physiological conditions of this new GPCR family, and further studies are required to evaluate our findings in endogenous expression systems. On the other hand,

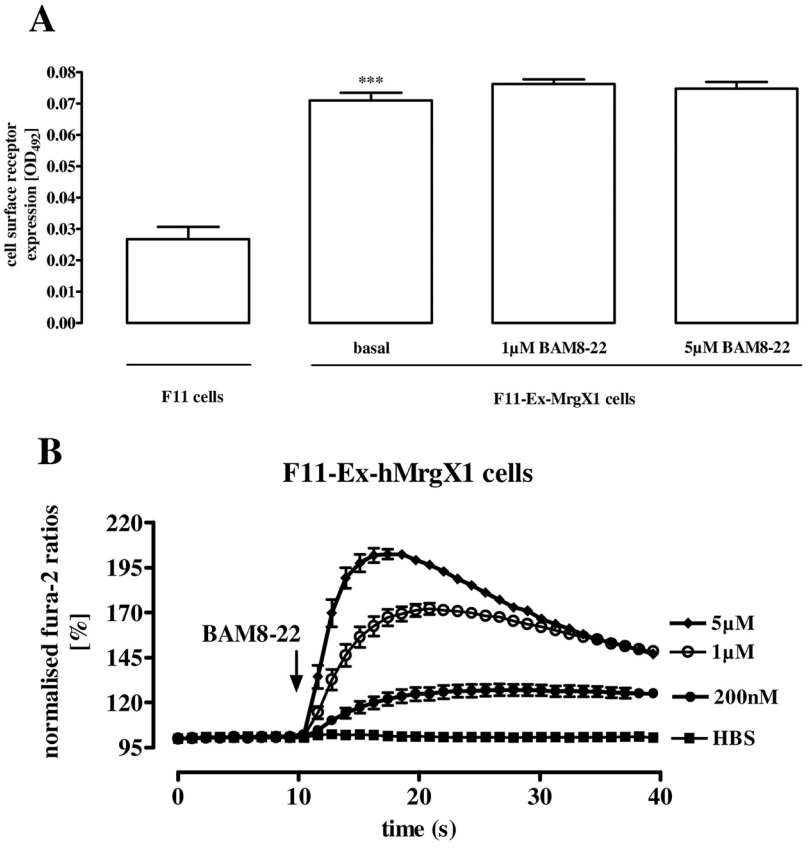

Fig. 6. Stable expression of human MrgX1 in F11 cells. A, receptor cell surface expression of control or with BAM8–22 (1 or 5 μM) -treated (30 min at 37°C) F11-Ex-hMrgX1 cells was monitored by the ELISA technique with intact cells. Absolute values of one (of four) representative experiment carried out in quadruplicate are shown. Asterisks indicate a significant (***, $p < 0.001$) difference between mock-transfected F11 and F11-Ex-hMrgX1 cells. Data are shown as the mean ± S.E. B, calcium signals in fura-2-loaded F11-Ex-hMrgX1 cells after injection of indicated concentrations of BAM8–22 at time point 10 s were measured. As a negative control, only HBS buffer was injected. Data were normalized by defining the first ratio (1.14 s) measured as 100%. Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate.

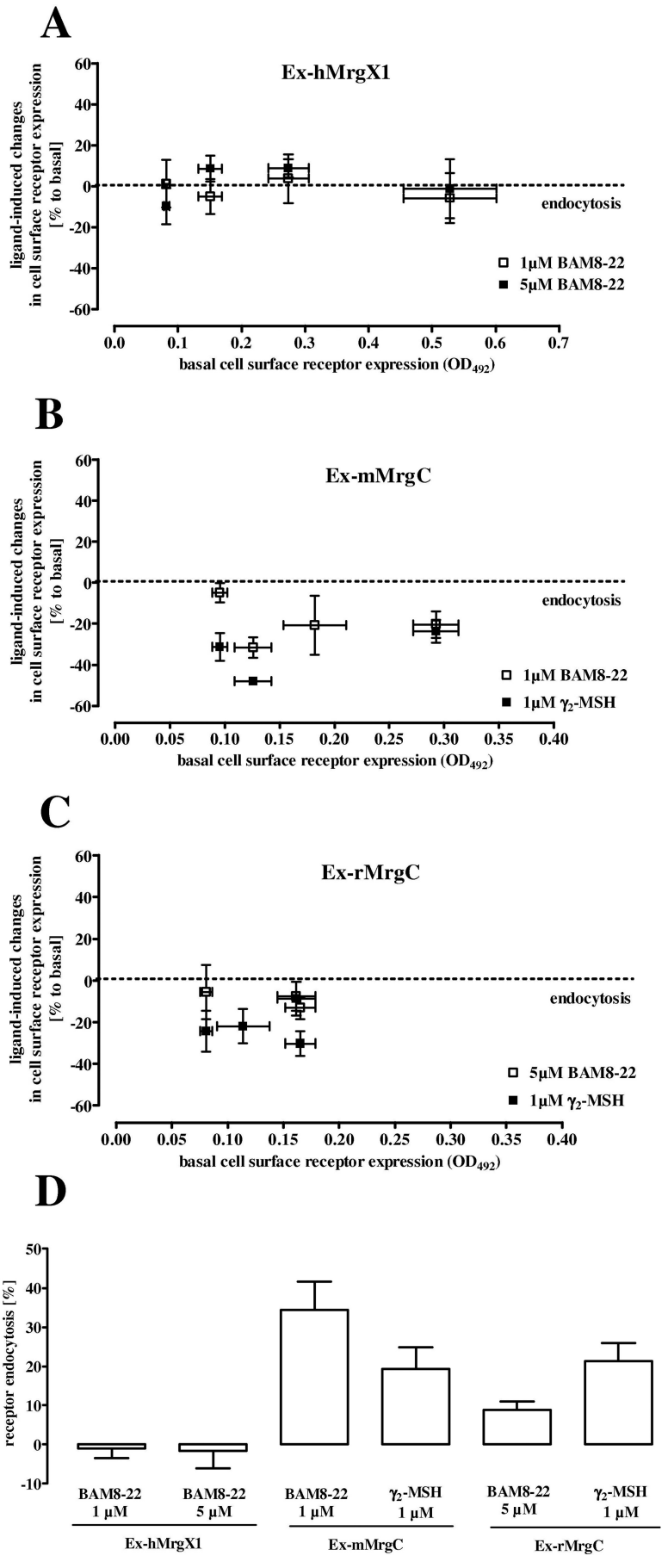

Fig. 7. Agonist-promoted endocytosis of Mrg receptors in ND-C cells. A to C, cell surface receptor expression under basal conditions (*x*-axis) monitored by the ELISA technique with intact cells is plotted against ligand-induced changes in cell surface receptor expression (*y*-axis). A, Ex-hMrgX1 expressing cells were stimulated with 1 μM BAM8–22 (□) or 5 μM (■). B, Ex-mMrgC-expressing cells were stimulated with 1 μM BAM8–22 (□) or 1 μM γ_2-MSH (■). C, Ex-rMrgC expressing cells were stimulated with 5 μM BAM8–22 (□) or 1 μM γ_2-MSH (■). Incubations were carried out for 30 min at 37°C. Background values of mock-transfected cells have been subtracted. D, the same data are presented as ligand-promoted endocytosis as percentages. Results are expressed as the mean ± S.E. of four independent experiments carried out in quadruplicate.

although resistance of hMrgX1 to endocytosis is uncommon among GPCRs, it has been described for a few members of this protein family, including the β_3-AR (Nantel et al., 1993), the somatostatin-4 (Schreff et al., 2000), or the κ-opioid receptor (Chu et al., 1997). Absence of β_3-AR desensitization, first found in recombinant expression systems (Nantel et al., 1993), was confirmed in an endogenous cell system (Jockers et al., 1998). Similar findings were reported for the κ-opioid (Wang et al., 2008) and the somatostatin-4 receptor (Schreff et al., 2000), indicating that lack of receptor endocytosis is not an artifact per se because of heterologous protein expression.

It is noteworthy that Lembo et al. (2002), contemporaneously to Dong et al. (2001), discovered a new receptor family that is exclusively expressed in small-diameter DRG neurons and therefore is known as the "sensory neuron-specific G protein-coupled receptor" (SNSR) (Lembo et al., 2002). Six members of the SNSR subfamily (SNSR1–6) were described with SNSR1 being identical with the hMrgX3, SNSR6 to the hMrgX4, and SNSR4 to the hMrgX1 characterized herein. Of the remaining members of the SNSR family, only the SNSR3 (sometimes also termed hMrgX7) was further analyzed. It is noteworthy that this receptor subtype shares the highest sequence homology with the hMrgX1 and is also activated by BAM8–22 (Lembo et al., 2002). In fact, these two receptor subtypes are more than 98% identical on the protein level. Given the fact that endocytosis-resistant and -prone receptors are grouped within the same subfamily (e.g., adrenergic or opioid receptors), it will be an interesting task for future studies to analyze the response of the SNSR3 to prolonged agonist exposure.

In general, receptor activity-regulating processes, such as endocytosis, tend to limit the strength of cellular signaling and thus prevent overstimulation of the system. In the case of the aforementioned β_3-AR, it has been shown that absent receptor desensitization leads to long-lasting physiological effects compared with the β_2-AR (Trochu et al., 1999), proposing that resistance of receptor endocytosis prolongs the physiological effects of a given GPCR. Furthermore, receptor endocytosis has been shown to dramatically affect the pharmacodynamics of a given drug. In the case of opioids, for example, a strong correlation between tolerance or dependence and receptor endocytosis (relative activity versus endocytosis) has been proposed (Alvarez et al., 2001). In detail, Kim et al. (2008) reported recently that mice transgenetically expressing a mutant of the μ-opioid receptor, which is in contrast to the wild-type receptor prone to morphine-induced endocytosis resist the development of morphine tolerance. These data highlight the correlation between tolerance and receptor endocytosis and indicate that knowledge about agonist-promoted receptor endocytosis provides useful information to interpret or predict the actions of a given drug in vivo.

Depending on the physiological role of the hMrgX1 [algetic as described by Grazzini et al. (2004) or analgetic as described by Chen et al. (2006)] sustained signaling because of its resistance to endocytosis may have different consequences in vivo. Prolonged signaling of an endocytosis-resistant receptor that maintains an analgetic tonus may be favorable in a situation of long-term noxious stimulation. In this scenario, treatment of chronic pain with synthetic hMrgX1 agonists might benefit from long-lasting analgetic signaling, being advantageous over the established opioid-based therapy, which suffers a significant loss of efficiency partly because of receptor desensitization (Ueda and Ueda, 2009). Considering algetic effects, prolonged hMrgX1 signaling could be the cause of chronic pain, which could then be treated with specific antagonists or inverse agonists. In any case, the development of pain-controlling hMrgX1-specific drugs requires a suitable animal model for the testing and improvement of the pharmacodynamics of these substances. Assuming that species-specific differences in agonist-promoted Mrg endocytosis also occur in vivo, data of pain-controlling drugs obtained in the rodent model must be interpreted with great caution in regard to potentially prolonged or even altered modes of action when applied to humans.

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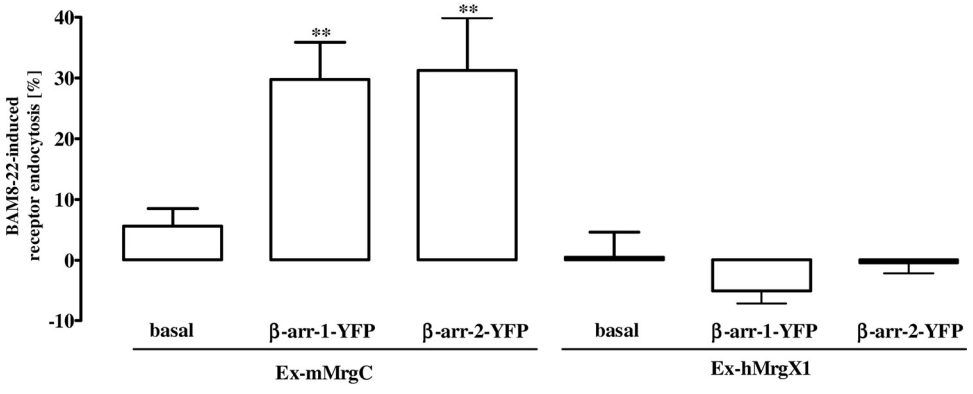

Fig. 8. β-Arrestin-dependent agonist-promoted mMrgC endocytosis in Cos cells. Ex-MrgX1 or Ex-mMrgC receptors were transiently expressed with or without β-arrestin-1-YFP or β-arrestin-2-YFP in Cos cells. Receptor cell surface expression of control or with BAM8–22 (1 μM, 30 min at 37°C) -treated cells was monitored by the ELISA technique with intact cells. BAM8–22-induced endocytosis of five independent experiments is given as a percentage. Background values of mock-transfected cells have been subtracted. Asterisks indicate a significant (**, $p < 0.01$) difference between β-arrestin coexpressing and solely receptor-expressing cells. Ex-MrgX1 or Ex-mMrgC expression varied from optical density readings between 0.07 and 0.74 (mean, 0.20) or 0.03 and 0.44 (mean, 0.19), respectively. The expression level of arrestin-YFP fusion proteins in Ex-MrgX1- or Ex-mMrgC-expressing cells was compared by measuring YFP fluorescence in a plate reader. No significant differences were observed.

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Address correspondence to: Dr. Andreas Breit, Walther-Straub-Institut für Pharmakologie und Toxikologie, Ludwig-Maximilians-Universität München, 80336 München, Germany. E-mail: andreas.breit@lrz.uni-muenchen.de