MRGPRX2 and Immediate Drug Hypersensitivity: Insights from Cultured Human Mast Cells

Short title: MRGPRX2 and drugs

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Abstract

Background: Mast cell (MC) degranulation via activation of the Mas-related G protein-coupled receptor X2 (MRGPRX2) is held to be of key importance for immediate drug hypersensitivity (IDH). However, data in humans are limited to observations in specific cell lines.

Objective: To study the usefulness of silencing MRGPRX2 in human MCs to further unveil the MRGPRX2 pathway in IDH.

Methods: MCs were cultured out of CD34+ progenitor cells obtained from peripheral blood (PBCMCs) and incubated with substance P as positive control, rocuronium, moxifloxacin, morphine or amoxicillin. Immunophenotyping of the cells included flow cytometric and microscopic analyses of the expression of CD117, CD203c and MRGPRX2. Intracellular calcium was measured using Fluo-4. Degranulation was analyzed by quantification of CD63 expression. For MRGPRX2 silencing, MCs were electroporated with Dicer-substrate silencing RNAs.

Results: Incubation of MCs with substance P, morphine and moxifloxacin increased intracellular calcium levels and triggered MC degranulation, which, for the drugs, is almost completely abolished by selective MRGPRX2 silencing. Despite an increase in intracellular calcium in MRGPRX2+ve cells, incubation with non-toxic concentrations of rocuronium does not result in degranulation of PBCMCs, whereas amoxicillin has no effect on PBCMCs.

Conclusion: The use of MRGPRX2 silencing in human MCs can provide important insights into the role of MRGPRX2 in the pathogenesis of IDH. As induction of calcium signals does not necessarily translate into a secretory response, measurement of the degranulation reaction seems more meaningful in the context of drug testing.

Resumen

Antecedentes: la desgranulación de los mastocitos (MC) a través de la activación del receptor X2 acoplado a proteína G relacionada con Mas (MRGPRX2) se considera clave para la hipersensibilidad inmediata a drogas (IDH). Sin embargo, los datos en humanos se limitan a observaciones en líneas celulares específicas.

Objetivo: Estudiar la utilidad del silenciamiento de MRGPRX2 en MC humanos para conocer mejor la vía MRGPRX2 en IDH.

Métodos: los MC se cultivaron a partir de células progenitoras CD34+ obtenidas de sangre periférica (PBCMC) y se incubaron con la sustancia P como control positivo, rocuronio, moxifloxacina, morfina o amoxicilina. El inmunofenotipaje de las células incluyó análisis por citometría de flujo y microscopia de la expresión de CD117, CD203c y MRGPRX2. El calcio intracelular se midió usando Fluo-4. La desgranulación se analizó por cuantificación de la expresión de CD63. Para el silenciamiento de MRGPRX2, los MC se electroporaron con ARN silente del sustrato Dicer.

Resultados: La incubación de MC con sustancia P, morfina y moxifloxacina provocó el aumento los niveles de calcio intracelular y desencadenó la desgranulación de MC, en el caso de la desgranulación provocada por las drogas esta eliminó casi por completo mediante el silenciamiento selectivo de MRGPRX2. A pesar del aumento del calcio intracelular en las células MRGPRX2+, la incubación con concentraciones no tóxicas de rocuronio no produce la desgranulación de los PBCMC, mientras que la amoxicilina no tiene efecto sobre los PBCMC.

Conclusión: El uso del silenciamiento de MRGPRX2 en MC humanos puede proporcionar información importante sobre el papel de MRGPRX2 en la patogénesis de IDH. Como la inducción de señales de calcio no se traduce necesariamente en una respuesta secretora, parece más significativa la medición de la reacción de desgranulación en el contexto de las pruebas a drogas.

Introduction

Much of what we know about the functionality of the Mas-related G protein coupled receptor X2 (MRGPRX2) has been elegantly demonstrated by Gaudenzio et al. [1] who compared the down-stream processes between cross-linking of IgE/FcεRI (high affinity receptor for IgE) and occupation of MRGPRX2 in mast cells (MC) derived from CD34+ progenitor cells. Since the description by Mc Neil et al. [2], increasing lines of evidence indicate that MC degranulation via occupation of MRGPRX2 constitutes a novel endotype of immediate drug hypersensitivity (IDH) independent from cross-linking of IgE/FcεRI complexes [1, 3-9]. Examples of drugs that act via MRGPRX2 are neuromuscular blocking agents (NMBAs), fluoroquinolones (FQs), icatibant and the opiate morphine. Many of these drugs harbour a tetrahydroisoquinoline (THIQ) motif [2].

However, the role and the relevance of MRGPRX2-mediated activation of human MCs by drugs have mainly been gathered from genetically modified animals and data in humans are limited to observations in specific cell lines. Moreover, translation of results obtained on the MRGPRB2 in the murine system to MRGPRX2 biology in humans is difficult [5, 8] and probably reflects the adaptive evolution of the human MRGPRX2 gene [10]. For example, Lansuet et al. [3] and Navínes-Ferrer et al. [8] failed to confirm that rocuronium, which causes mouse MC activation and degranulation, to activate human LAD2 MCs [2]. Alternatively, LAD2 MCs have been reported to show variable levels of MRGPRX2 expression, as compared to human mature skin MCs [11], which show high expression of the receptor [12, 13].

Recently, we optimized a method to culture human MC from peripheral blood CD34+ progenitor cells designated as PBCMCs [14]. These PBCMCs express CD117, CD203c, FcεRI, chymase, tryptase and histamine and respond to the neurokinin substance P, a natural MRGPRX2 agonist [14, 15]. Thus, this human MC model appears suitable for the
exploration of the MRGPRX2 pathway at single cell level by flow cytometry in IDH, especially if we would succeed in suppressing expression of the receptor via introduction of silencing RNA through transfection techniques[1, 16, 17] or via electroporation with dicer small interference RNA (DsiRNA)[18].

In light of these advances and given our experience with IDH to NMBAs [19-21], FQs[22, 23] and opiates [24, 25], here we silenced MRGPRX2 in human PBCMCs via DsiRNA electroporation to better define the underlying pathomechanisms of three drugs (rocuronium, moxifloxacin, morphine) that commonly cause IDH. In addition, we studied also amoxicillin, a β-lactam antibiotic that is known to activate MC through cross-linking of drug-specific IgE/FceRI[8].
Materials and methods

In vitro culture of human peripheral blood cultured mast cells (PBCMCs)

Human PBCMCs were cultured as described elsewhere[14]. Briefly, CD34^{+ve} progenitors were isolated using the EasySep Human CD34 Selection Kit (Stemcell Technologies) out of Histopaque-isolated peripheral blood mononuclear cells. Isolated CD34^{+ve} progenitors were cultured in a serum-free methylcellulose-based medium (MethoCult SF H4236, Stemcell Technologies) supplemented with penicillin (100 units/mL, Life Technologies), streptomycin (100 μg/mL, Life Technologies), low-density lipoprotein (LDL, 10 μg/mL, Stemcell Technologies), 2-mercaptoethanol (55 μmol/L, Life Technologies), stem cell factor (SCF, 100 ng/mL, MiltenyiBiotec) and interleukin-3 (IL-3, 100 ng/mL, PeproTech) during 4-5 weeks. All donors gave written informed consent as approved by the Ethical Committee of the Antwerp University Hospital (Belgium B300201837509).

Intracellular calcium staining

PBCMC, defined as cells expressing both CD117 and CD203c, were functionally studied before and after activation using two techniques, i.e., staining of intracellular calcium with Fluo-4 AM (ThermoFisher Scientific) and quantification of the lysosomal degranulation marker CD63. For intracellular calcium staining, PBCMCs, at a concentration of 5x10^5 cells/mL, were loaded with 1 μM Fluo-4 AM for 45 min at 37°C. After staining, the cells were washed with PBS (ThermoFisher Scientific) and resuspended in 300 μL pre-warmed (37°C) Tyrode’s buffer (Sigma-Aldrich). The intensity of Fluo-4 AM was measured at single cell level for 50 sec without stimulation, next the cells were stimulated with Tyrode’s buffer as negative control, substance P (74 μM, Sigma Aldrich) as positive control, amoxicillin (GSK), morphine hydrochloride (Sterop), moxifloxacin hydrochloride (Sigma Aldrich), rocuronium bromide (Esmeron®; Organon) or anti-FcεRI (2.5 μg/mL, ThermoFisher Scientific) followed by
Immediately further reading for 2 min. Dose-response experiments implied final concentrations of amoxicillin (137 µM, 684 µM and 1370 µM; n=3), morphine hydrochloride (50 µM, 250 µM and 500 µM; n=10), moxifloxacin hydrochloride (11.4 µM, 114 µM and 571 µM; n=10) or rocuronium bromide (1.64 µM, 16.4 µM, 164 µM, 1640 µM, 3280 µM and 8200 µM; n=10). Optimal stimulation concentration were determined as followed: amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM, as the higher concentrations for rocuronium appeared to be cytotoxic). Combining the staining of MRGPRX2 and intracellular calcium comprised first staining of the membrane expression of MRGPRX2 for 20 min at 4°C, after which the cells were washed with PBS and stained with Fluo-4 AM according to the protocol described above.

**CD63 up-regulation**

For CD63 measurement, PBCMCs were dissolved in pre-warmed (37°C) Tyrode’s buffer at a concentration of 5x10^5 cells/mL. Next, 100 µL of the cells were stimulated with 100 µL amoxicillin, morphine hydrochloride, moxifloxacin hydrochloride, rocuronium bromide or anti-FceRI, at the above-described final concentrations, or with 100 µL of Tyrode’s buffer as a negative control and 100 µL of substance P as a positive control, for 3 and 20 min at 37°C. Reactions were stopped by placing the cells on ice and subsequently the supernatants is removed after centrifugation (500 x g, 4°C, 5 min). Cells were stained with anti-CD117-APC (clone 104D2, BD Biosciences), anti-CD203c-PECy7 (clone NP4D6, eBioscience), anti-human MRGPRX2-PE (clone K125H4, BioLegend) and anti-CD63-FITC (clone H5C6, BD Biosciences) for 20 min at 4°C. Next, cells were fixed with 1 mL Phosflow Lyse/Fix buffer (BD Biosciences) for 20 min. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured using flow cytometry.
Flow cytometric analyses

Flow cytometric analyses was performed on a calibrated FACSCalibur (BD Immunocytometry systems) with argon-ion lasers (488nm and 633nm), for the intracellular calcium measurements, or on a calibrated FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with three lasers (405 nm, 488 nm and 633 nm), for the CD63 measurement. Correct compensation settings were performed using BD CompBeads (BD Biosciences). Flow cytometric data were analysed using Kaluza Analysis 2.1 software (Beckman Coulter) and FCS6 Express 6 flow research edition (de novo software, Glendale, California). A fluorescence minus one (FMO) sample was used to set a marker between positive and negative cells according to the 99th percentile. The results of the calcium measurements were expressed as fold increase against the basal intensity. Results of CD63 measurements were expressed as the net value of percentages of positive cells, that is percentage of CD63 positive in stimulated cells minus percentage CD63 positive in resting cells. At least 500 PBCMCs, gated as CD117+CD203c+, were counted per sample.

DsiRNA electroporation

Human MCs at a concentration of 1x10^6 cells/mL were washed twice in cold serum-free Opti-MEM I medium (Gibco Invitrogen) and resuspended in 200 µL of the same medium. Cells were transferred to a 4.0-mm electroporation cuvette (Cell Projects) and 1 µM pool of two DsiRNA against MRGPRX2 at a 1:1 ratio or a non–targeting control DsiRNA (Integrated DNA Technologies, Catalog #:51-01-14-03) were added to the cuvette (Duplex sequences: DsiRNA 1: 5’-GGCAUUCAGGGGUUCCUAUAAT-3’ and 3’-AACCGUAAGUCACCAAGGAUAUAUA-5’, DsiRNA 2: 5’GUUACGUGUCCA CAGAAUUAAATA-3’ and 3’-UUCAAUGCACAAGGUGUCUUUAUUUAU-5’). A square wave protocol (500 V, 5 ms, 0 gap, 1 pulse) was used to electroporate the cells (Gene
PulserXcell\textsuperscript{TM} device, Bio-Rad Laboratories). Immediately after electroporation cells were transferred to 5 mL of IMDM medium supplemented with 10\% FBS (pre-heated at 37°C) and incubated for 20 min at 37°C and 5\% CO\textsubscript{2}. Thereafter, cells were centrifuged and transferred to SCF-medium. Five days after electroporation, a repeat analyses of expression and functionality of the PBCMC was performed as described above. The five days was determined after performing a follow-up analyses of the expression on different time point after electroporation. Transfection efficiency was examined by electroporating the cells with 1 µg of mRNA for enhanced green fluorescent protein (EGFP) per 10\textsuperscript{6} cells. mRNA against EGFP was made as described elsewhere\cite{26}. Briefly, SoloPack Golden supercompetent E. coli cells were transformed with pGEM-4Z DNA plasmids. The transformed E. coli cells were cultured and amplified in LB-ampicillin agar plates and LB-ampicillin cultures, respectively. Plasmid DNA isolation and purification were performed using the NucleobondXtra Midi EF and Nucleobond finalizer kits (Macherey-Nagel). The isolated DNAs were digested with SpeI restriction enzyme (Thermo Fisher Scientific). Capped mRNA transcripts were synthesized from linearized plasmids and purified by DNase digestion and LiCl precipitation using a mMessagemMachine T7 \textit{in vitro} transcription kit (Life Technologies).

\textit{Microscopic analyses}

First, PBCMCs were purified with the Easysep\textsuperscript{TM} Dead Cell Removal ( Annexin V) Kit (Stemcell\textsuperscript{TM} Technologies). The purified PBCMC’s were then loaded with 1 µM Fluo-4 AM for 45 min at 37°C. After staining, the cells were washed with PBS, resuspended in preheated (37°C) Tyrode’s buffer (Sigma-Aldrich) and plated on Poly-D-Lysin-coated Nunc\textsuperscript{TM} LabTek\textsuperscript{TM} Chambered Coverglass (ThermoFisher Scientific) at 37°C. When the cells were attached to the coverglass, anti-CD63 Alexa Fluor\textsuperscript{TM} 647 (clone H5C6, BD Biosciences) was added and cells were stimulated with buffer, substance P (74 µM), amoxicillin (1370 µM),
morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM) and imaged every 5 sec using a Leica TCS SP8 confocal microscope with environmental chamber. For calcium analysis, the HC PL APO CS2 20x/0.75 IMM objective was used. For representative movies, the HC PL APO CS2 63x/1.40 OIL objective was used. Further analysis was done with Leica LAS X software and Image J.

Statistical analysis

GraphPad Prism version 7 software was used for data analysis and paired Student’s t-tests were performed. Results are expressed as mean ± SEM. A P-value of < 0.05 was considered significant. The “n” in the figures denotes the total number of different donors used.

Results

PBCMC were cultured out of peripheral blood of 17 healthy volunteers without drug hypersensitivity. Human PBCMCs are defined as viable cells expressing both CD117 and CD203c[15]. As shown in figure 1 of the Online Repository, PBCMCs harbour two distinct subpopulations, i.e., a subpopulation expressing MRGPRX2(MRGPRX2+ve) and a subpopulation negative for the MRGPRX2(MRGPRX2-ve). Each culture yields MRGPRX2+ve cells with a mean expression of 52 ± 3% MRGPRX2+ve cells (n=17), allowing comparative analyses. Preliminary dose-response curve studies for the evaluated drugs were used to determine the optimal stimulation concentration and are shown in Figure 2 of the Online Repository. For substance P, stimulation conditions are detailed elsewhere[15]. The optimal concentrations are described in the methods’ section.

The kinetics of changes of intracellular calcium levels after stimulation of PBCMCs are displayed in figure 1 and representative plots are shown in figure 3 of the Online Repository also the movies 1-4 shows these results. Stimulation with substance P, morphine and
moxifloxacin results in a rapid increase in intracellular calcium, which peaks within approximately 75 sec and slowly fades without returning to baseline after 3 min. Stimulation with rocuronium at a maximum non-toxic concentration enhances intracellular calcium levels, but this elevation is less pronounced and almost returns to baseline levels after 3 min. Amoxicillin does not induce changes in intracellular calcium. Figure 2 of the main text and figure 4 of the Online Repository show that intracellular calcium responses to the drugs are restricted to the MRGPRX2\(^{\text{+ve}}\) cells. In contrast, for substance P calcium mobilization is also observed in MRGPRX2\(^{\text{-ve}}\) cells, which is less pronounced compared to the MRGPRX2\(^{\text{+ve}}\) cells.

Surface expression of the lysosomal degranulation marker CD63 is shown in figure 3. No surface expression of CD63 is observed in cells stimulated with rocuronium or amoxicillin. Likely, these observations reflect insufficient or absent calcium mobilization, respectively. In contrast, a clear up-regulation of CD63 is seen when PBCMCs are incubated with substance P, morphine or moxifloxacin (movies 1-4). For the drugs, paralleling the findings of the calcium staining experiments, degranulation, as reflected by the up-regulation of CD63, is restricted to MRGPRX2\(^{\text{+ve}}\) cells. Moreover, this up-regulation was most pronounced in the cells expressing highest baseline MRGPRX2 density on their plasma membrane. In contrast, substance P induces calcium mobilization in MRGPRX2\(^{\text{+ve}}\) and in a lesser extent also in MRGPRX2\(^{\text{-ve}}\) cells. Degranulation to the neurokinin is restricted to the MRGPRX2\(^{\text{+ve}}\) subpopulation, likely because of insufficient calcium mobilization in the MRGPRX2\(^{\text{-ve}}\) cells (Figure 3B).

To confirm the role of MRGPRX2, we applied a silencing technique using electroporation to allow the entrance of DsiRNA\([18, 26]\). As shown in figure 5 of the Online Repository, based on the EGFP expression, this approach has a transfection efficiency in our PBCMCs of 60-90\% \((n=3)\). In this knock-down model, de novo expression of MRGPRX2 is markedly down
regulated, causing a reduction in surface levels of the receptor. MRGPRX2-DsiRNA-treated cells show a \(77 \pm 4\%\) decrease in MRGPRX2 expression compared to cells electroporated with the control DsiRNA. The maximal expression of FcεRI remains unaltered in both conditions (figure 6 of the Online Repository).

Functionally, introduction of MRGPRX2-DsiRNA almost completely suppressed intracellular calcium elevations in response to morphine, moxifloxacin and rocuronium, and in a lesser extent also to the neurokinin substance P. Moreover, for substance P, morphine and moxifloxacin this suppressive effect was also accompanied by a significant downscaling of CD63 up-regulation. In other words, the effect of MRGPRX2 silencing align with the differences observed between MRGPRX2+ve and MRGPRX2-ve cells as shown earlier in figures 2 and 3. MRGPRX2 silencing had no significant effect on anti-FcεRI-dependent intracellular calcium signaling and degranulation (expressed as CD63 up-regulation) in PBCMCs (Figure 4 in the main text and Figure 7 of the Online Repository).

**Discussion**

Activation of MRGPRX2, which is constitutively expressed by human skin MCs[13], is held to constitute a novel endotype of IDH that is clinically indistinguishable from MC degranulation in response to drug-specific cross-linking of IgE/FcεRI complexes[1-8]. However, support for the MRGPRX2 pathway in IDH is not uniform and uncertainties remain, since much of our knowledge relies on observations from calcium imaging techniques and findings in genetically modified animals and LAD2 cells. Here, we took advantage of our expertise to culture and gate sufficient numbers of human MC obtained out of peripheral blood progenitors of healthy donors (designated as PBCMCs)[14, 15] and DsiRNA electroporation to further explore the potential of certain drugs to act as MRGPRX2-dependent MC secretagogues. Our results demonstrate that the combined use of human MCs,
DsiRNA electroporation-based down regulation of MRGPRX2 and multicolour flow cytometry is an effective model for the identification of MRGPRX2-dependent MC activators. Moreover, as acknowledged by Folkerts et al. [17], cytometry techniques, might circumvent the limitation of single-cell imaging system, that is, detection of limited changes in protein expression changes or changes that have only subtle effects on MC activation and degranulation. Besides, we confirm that selective MRGPRX2 silencing does not affect MC activation/degranulation in response to cross-linking of IgE/FcεRI complexes [17]. Finally, we provide a proof-of-concept that our approach can eliminate some of the uncertainties and can deepen our mechanistic understandings of the MRGPRX2-related endotype of IDH. Importantly, we show that morphine, moxifloxacin and rocuronium, but not amoxicillin, induce a MRGPRX2-dependent rise of intracellular calcium, which is accompanied by degranulation, expressed as CD63 up-regulation, for morphine and moxifloxacin. Moreover, for morphine and moxifloxacin this calcium mobilization and degranulation is almost completely abolished by selective MRGPRX2 silencing. In line with the observation substance P also to trigger some MRGPRX2-ve cells to increase intracellular calcium levels, MRGPRX2 silencing did not completely suppress calcium mobilization by this neurokinin. Likely because of the presence of other neurokinin receptors on MC [27, 28] that are not suppressed by our silencing technique.

The dissociation between calcium signals and degranulation after stimulation with a maximum non-toxic concentration for rocuronium is intriguing. Although we cannot exclude this phenomenon to results from sub-optimal stimulation (as seen for morphine), it might call into question the contribution of calcium imaging techniques in exploring the potential of a drug to elicit IDH reactions via occupancy of MRGPRX2. Furthermore, the lack of degranulation with rocuronium is in contrast with findings in mice, but is in agreement with observations in other human cell line models, reemphasizing the notion that murine models...
may not always be suitable for human studies on MRGPRX2-mediated effects on MCs. Finally, our observations with moxifloxacin call for caution in the interpretation of negative findings obtained with LAD2 cells[3].

FQs can cause severe IDH reactions that are difficult, if at all, to document because of uncertainties associated with these non-specific histamine releasers in skin testing [22, 29, 30], drug-specific IgE assays [31, 32] and basophil activation tests (BAT)[23, 32-35]. The frequent occurrence of FQ IDH in drug naïve patients [30, 36] suggests that the underlying mechanism does not necessitate prior sensitization and makes occupancy of MRGPRX2 a likely mechanistic explanation [2, 6, 23]. We demonstrate that moxifloxacin can elicit a rise in intracellular calcium associated with degranulation of MRGPRX2+[PBCMCs and that these processes are abrogated by selectively silencing of MRGPRX2 via DsiRNA electroporation. These findings are in line with the observations reported by others [2, 5], i.e. that moxifloxacin (amongst other FQs) potentially leads to IDH via occupancy of the MRGPRX2. The earlier failure of LAD2 cells to reveal MRGPRX2 activation by moxifloxacin [3] could relate to the variable differentiation status of this cell line, limiting its use as a surrogate for human skin MCs [11].

NMBAs have long been identified as a principal cause of perioperative anaphylaxis [21, 37]. In most epidemiological surveys, skin tests have been considered to be specific for IgE/FcεRI-dependent reactions, whereas IgE/FcεRI-independent IDHR from N MBA shave been believed to yield negative skin test responses. However, in the light of the observations by McNeil et al.[2], it has been suggested to critically verify the value of skin testing and to reclassify anaphylaxis from N MBA [38]. In fact, the authors hypothesize that most NMBAs may cause non-IgE/FcεRI-mediated IDH rather than IgE/FcεRI-dependent IDH. However, our current observations argue against a too generalized mechanistic reclassification of N MBA hypersensitivity reactions.
Rocuronium elicits a relatively limited and transient elevation of intracellular calcium in MRGPRX2$^{\text{+ve}}$ cells, but these changes are not associated with MC degranulation. This observation parallels the negative findings of Lansuet al.[3] and Navinés-Ferrer et al.[8] using a human cell line model. The discrepancy between murine and human MCs related to MRGPRB2 and MRGPRX2, respectively, is best explained by adaptive changes of the MRGPRX2 gene in human evolution [10], making the human receptor more than 10-fold less susceptible to rocuronium than its murine orthologue [2]. Moreover, our experimental data match the outcomes of quantification of specific IgE (sIgE), BAT and skin testing in the vast majority of our patients, indicating that rocuronium anaphylaxis predominantly results from IgE/FceRI cross-linking[21]. Admittedly, most patients who suffer from rocuronium anaphylaxis are also drug naïve. Prior sensitization in these patients is likely to result from cross-reactivity towards household and other environmental agents and medicines [39] that harbour structurally similar epitopes, especially tertiary and quaternary substituted ammonium structures [40] or compounds that might trigger excessive non-allergen-specific IgE production such as pholcodine[41]. Of note, the finding of calcium staining to dissociate with up-regulation of CD63 calls for further investigation with other lysosomal-associated membrane proteins and mediator release to explore the functional consequences of calcium changes in response to rocuronium.

As reviewed by Baldo et al.[42], IDH reactions from opiates predominantly result from non-specific mediator release. The correct diagnosis of rare IgE/FceRI-dependent IDH to these drugs is difficult, mainly because of the absence of reliable drug-specific IgE assays [25] and uncertainties associated with non-specific skin testing [43]. Although the first description of morphine to activate MRGPRX2 dates back to2007[44], involvement of this receptor in opiate hypersensitivity has been suggested much more recently [3]. Here, we confirm that MRGPRX2 can be implicated in morphine-induced IDH[3, 8], as the drug induces elevated
intracellular calcium levels and degranulation of human MRGPRX2\(^{++}\)MCs, degranulation that can be inhibited by selective silencing of the receptor. Our data also explain why skin tests are not suited to distinguish between IgE/FcεRI-dependent and –independent hypersensitivity to opiates[13, 43]. Because of this limitation, and considering the poor reliability of opiate sIgE tests [25, 39], we think that, apart from drug provocation tests, only basophil activation experiments can contribute to correct diagnosis of IgE/FcεRI opiate hypersensitivity and help prevent erroneous over diagnosis[24, 25]. Although basophils were recently reported to constitutively express MRGPRX2[45], this finding is controversial [46, 47]. Consistent with the general assumption that IDH to amoxicillin is mainly IgE/FcεRI-dependent[8], we could not elicit MRGPRX2-mediated MC activation/degranulation with this \(\beta\)-lactam antibiotic. However, in line with earlier observations with proteinaceous allergens [15] and our recent experience with chlorhexidine [48], it is attractive to speculate that our MCs might become responsive to this \(\beta\)-lactam antibiotic after passive sensitization with sera from patients who experienced an IgE/FcεRI-dependent amoxicillin allergy.

Although our experiments add to the knowledge about MRGPRX2-dependent drug hypersensitivity, the observation that only a minority of the patients experiences such reactions and that these reactions generally to be restricted to a particular drug (or drug class) remains a pathomechanistic conundrum. As elegantly addressed by Porebski et al. [6], one could hypothesize these observations to relate to genetic polymorphisms and mutations resulting in an augmented responsiveness of the receptor, distinct receptor binding sites, differences in MRGPRX2 signal some, epigenetic modifications, post-transcriptional modifications resulting in synthesis of MRGPRX2 variants, temporarily or constitutively varying surface expressions and even the influence of co-factors. In this context, a limitation in our method, as in many in vitro and ex vivo experiments, lies in the necessity of supra-therapeutic (sometimes near toxic as seen for rocuronium) stimulation concentrations that
might blunt subtle differences in affinity or avidity of the molecules for MRGPRX2. Perhaps, direct comparisons between compounds harbouring different numbers of tetrahydroisoquinolines can, to some extent, shed light.

Taken together, comparative analyses of human MRGPRX2+ve and MRGPRX2−ve MCs and selective silencing of MRGPRX2 via DsiRNA electroporation coupled to flow cytometry can clarify some uncertainties and improve our understandings of the MRGPRX2 pathway in IDH. Our data call for prudent interpretation of changes in intracellular calcium, in the field of IDH, as induction of calcium signals does not necessarily translate into a degranulation. The use of this approach may eventually call for the reclassification of IDH in IgE/FceRI-dependent and MRGPRX2-dependent reactions amongst others (with largely unknown mechanism). Future studies should use this model to assess the effects of MRGPRX2-silencing on responses of activated MCs other than degranulation markers, such as the generation and release of lipid mediators, cytokines and vascular endothelial growth factor (VGEF)[1] and to compare this approach to other means of identifying human MC degranulators[17]. The use of our model to improve our understanding of the different pathomechanisms of IDH could culminate in the development of novel diagnostics and therapeutic options including MRGPRX2 antagonists for the prevention and treatment of IDH.

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Conflict of interest

M. Maurer has received honoraria (advisory board, speaker) and/or institutional grant/research support from Allakos, Amgen, Astra-Zeneca, Bayer, Dr.Pfleger, FAES, Genentech, GSK, Innate Pharma, Kyowa Kirin, Lilly, Merckle Recordati, Moxie, Novartis, Regeneron, Roche, Sanofi, MSD, UCB, and Uriach. The other authors declare no competing financial interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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References


Figures

Figure 1: Calcium imaging of human PBCMC.

(A) Flow cytometric data and (B) microscopy data. Incubation with buffer, the positive control substance P (74 µM), the natural agonist of MRGPRX2, amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM). Fold increase is calculated against the basal intensity.
Figure 2: Flow cytometric calcium imaging in MRGPRX2\(^{+}\)ve (black) and MRGPRX2\(^{-}\)ve (red) subpopulations.

PBCMC samples were (after 50 sec) incubated with buffer, the natural ligand of MRGPRX2 substance P (74 \(\mu\)M), amoxicillin (1370 \(\mu\)M), morphine (500 \(\mu\)M), moxifloxacin (571 \(\mu\)M) or rocuronium (1640 \(\mu\)M). In all experiments, \(n=3\). Area under the curves were compared using a paired student t-test, \(p<0.05^*, p<0.01^{**}\). Note that in the absence of MRGPRX2, there is no calcium response to the drugs, while there is a low response to the neurokinin substance P.
(A-B) Flow cytometric data and (C) microscopy data. (A) Time curves of CD63 up-regulation. (B) Representative plots for CD63 up-regulation. (C) Representative still frames of live cell imaging showing intracellular calcium staining (pseudocolor) and extracellular CD63 (red - arrow) up-regulation in PBCMC. PBCMCs were incubated for 3 min with buffer, substance P (74 µM), amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM).
Figure 4: Effect of MRGPRX2 silencing on PBCMC functionality after MRGPRX2-specific DsiRNA electroporation.

PBCMCs were electroporated with a negative control DsiRNA (black) or a MRGPRX2-specific DsiRNA (red). (A) Effect of the silencing on the calcium levels and (B) CD63 up-regulation after 3 min of incubation. Incubation with substance P (74 µM), the natural agonist of MRGPRX2, anti-FcεRI (2.5 µg/mL), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM). In all experiments, n=4. Area under the curves were compared using a paired student t-test, p < 0.05*, p < 0.01**, p < 0.001***. Note that the effect of MRGPRX2 silencing align with the differences observed between MRGPRX2⁺ve and MRGPRX2⁻ve cells as shown in figures 2 and 3.