ORIGINAL ARTICLE

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

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Abstract

Background: Mast cell (MC) degranulation via activation of the Mas-related G protein–coupled receptor X2 (MRGPRX2) plays a key role in 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 with the aim of further unveiling the MRGPRX2 pathway in IDH.
Methods: MCs were cultured from CD34$^+$ progenitor cells obtained from peripheral blood (PBCMCs) and incubated with substance P (as a positive control), rocuronium, moxifloxacin, morphine, or amoxicillin. Immunophenotyping of the cells included flow cytometry and microscopy analyses of the expression of CD117, CD203c, and MRGPRX2. Intracellular calcium was measured using Fluo-4. Degranulation was analyzed by quantifying CD63 expression. For MRGPRX2 silencing, MCs were electroporated with Dicer small interference 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+ cells, incubation with nontoxic concentrations of rocuronium does not result in degranulation of PBCMCs. 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.

Key words: Amoxicillin. Calcium. CD63. Flow cytometry. Mast cells. Morphine. Moxifloxacin. MRGPRX2. Rocuronium.

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 fármacos. 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 la hipersensibilidad inmediata a fármacos.
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 de los niveles de calcio intracelular y desencadenó la desgranulación de MC. En el caso de la desgranulación provocada por los fármacos, ésta se 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 la hipersensibilidad inmediata a fármacos. 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 fármacos.

Palabras clave: Amoxicilina. Calcio. CD63. citometría de flujo. Mastocitos. Morfina. Moxifloxacino. MRGPRX2. Rocuronio.

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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 downstream processes between cross-linking of IgE/FcεRI (high affinity receptor for IgE) and occupation of MRGPRX2 in mast cells (MCs) derived from CD34$^+$ progenitor cells. Since the description by McNeil et al [2], increasing lines of evidence indicate that MC degranulation via occupation of MRGPRX2 constitutes a novel endotype of immediate drug hypersensitivity (IDH) that is independent from cross-linking of IgE/FcεRI complexes [1,3-9]. Examples of drugs that act via MRGPRX2 are neuromuscular blocking agents (NMBAs), fluoroquinolones, icatibant, and the opiate morphine. Many of these drugs harbor a tetrahydroisoquinoline motif [2].

The role and the relevance of MRGPRX2-mediated activation of human MCs by drugs have mainly been ascertained from genetically modified animals, and data in humans are limited to observations in specific cell lines. Moreover, translation of results obtained on 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, Lansu et al [3] and Navinés-Ferrer et al [8] failed to confirm that rocuronium, which causes mouse MC activation and degranulation, activates human LAD2 MCs [2]. Alternatively, LAD2 MCs have been reported to show variable levels of MRGPRX2 expression, compared with human mature skin MCs [11], which show high expression of the receptor [12,13].

We recently optimized a method to culture human MCs from peripheral blood CD34$^+$ progenitor cells (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 succeed in suppressing expression of the receptor via RNA silencing 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], fluoroquinolones [22,23], and opiates [24,25], we silenced MRGPRX2 in human PBCMCs via DsiRNA electroporation to better define the underlying pathogenic mechanisms of 3 drugs (rocuronium, moxifloxacin, morphine) that commonly cause IDH. We also studied amoxicillin, a β-lactam antibiotic that is known to activate MC through cross-linking of drug-specific IgE/FcεRI [8].

Materials and Methods

In Vitro Culture of Human PBCMCs

Human PBCMCs were cultured as described elsewhere [14]. Briefly, CD34$^+$ progenitors were isolated using the EasySep Human CD34 Selection Kit (Stemcell Technologies) from Histopaque-isolated peripheral blood mononuclear cells. Isolated CD34$^+$ 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, Miltenyi Biotec), and interleukin 3 (IL-3, 100 ng/mL, PeproTech) for 4-5 weeks. All donors gave their written informed consent as approved by the Ethics Committee of the Antwerp University Hospital (Belgium B300201837509).

Intracellular Calcium Staining

PBCMCs, defined as cells expressing both CD117 and CD203c, were functionally studied before and after activation using 2 techniques, ie, 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 5×10^5 cells/mL were loaded with 1 µM Fluo 4 AM for 45 minutes at 37°C. After staining, the cells were washed with phosphate-buffered saline (PBS, ThermoFisher Scientific) and resuspended in 300 µL of prewarmed (37°C) Tyrode buffer (Sigma-Aldrich). The intensity of Fluo-4 AM was measured at single cell level for 50 seconds without stimulation. The cells were then stimulated with Tyrode buffer as a negative control, substance P (74 µM, Sigma Aldrich) as a positive control, amoxicillin (GSK), morphine hydrochloride (Sterop), moxifloxacin hydrochloride (Sigma Aldrich), rocuronium bromide (Esmeron; Organon), and anti-FcεRI (2.5 µg/mL, ThermoFisher Scientific) followed immediately by further reading for 2 minutes. Dose-response experiments involved 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), and rocuronium bromide (1.64 µM, 16.4 µM, 164 µmM, 1640 µM, 3280 µM, and 8200 µM; n=10). Optimal stimulation concentrations were determined, as follows: amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM), and rocuronium (1640 µM, as higher concentrations for rocuronium appeared to be cytotoxic). Combining the staining of MRGPRX2 and intracellular calcium comprised staining of the membrane to determine expression of MRGPRX2 for 20 minutes at 4°C, after which the cells were washed with PBS and stained with Fluo-4 AM according to the protocol described above.

Upregulation of CD63

For measurement of CD63, PBCMCs were dissolved in prewarmed (37°C) Tyrode buffer at a concentration of 5×10^5 cells/mL. Next, 100 µL of the cells was stimulated with 100 µL of amoxicillin, morphine hydrochloride, moxifloxacin hydrochloride, rocuronium bromide, and anti-FcεRI at the aforementioned final concentrations, or with 100 µL of Tyrode buffer as a negative control and 100 µL of substance P as a positive control for 3 and 20 minutes at 37°C. Reactions were stopped by placing the cells on ice. Subsequently, supernatants were removed after centrifugation (500 × g, 4°C, 5 minutes). Cells were stained with anti-human

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CD117-APC (clone 104D2, BD Biosciences), antihuman CD203c-PECy7 (clone NP4D6, eBioscience), antihuman MRGPRX2-PE (clone K125H4, BioLegend), and antihuman CD63-FITC (clone H5C6, BD Biosciences) for 20 minutes at 4°C. Next, cells were fixed with 1 mL Phosflow Lyse/Fix buffer (BD Biosciences) for 20 minutes. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured using flow cytometry.

Flow Cytometry Analysis

Flow cytometry analysis was performed on a calibrated FACSCalibur device (BD Immunocytometry Systems) with argon-ion lasers (488 nm and 633 nm) for the intracellular calcium measurements or on a calibrated FACSCanto II flow cytometer (BD Immunocytometry Systems) equipped with 3 lasers (405 nm, 488 nm, and 633 nm) for the CD63 measurements. Correct compensation settings were established using BD CompBeads (BD Biosciences). Flow cytometry data were analyzed using Kaluza Analysis 2.1 (Beckman Coulter) and FCS6 Express 6 (research edition) (De Novo Software). A fluorescence minus one 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 baseline intensity. The results of CD63 measurements were expressed as the net value of percentages of positive cells, that is, the percentage of $CD63^+$ cells in stimulated cells minus the percentage of $CD63^+$ cells in resting cells. At least 500 PBCMCs, gated as $CD117^+CD203c^+$, were counted per sample.

DsiRNA Electroporation

Human MCs, at a concentration of 1×10^6 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 a 1-µM pool of 2 DsiRNAs against MRGPRX2 at a 1:1 ratio or a nontargeting control DsiRNA (Integrated DNA Technologies, Catalog #:51-01-14-03) was added to the cuvette (Duplex sequences: DsiRNA 1, 5'-GGCAUUCAGUGGUUCCUAAUAUUAT-3' and 3'-AACCGUAAGUCACCAAGGA UUAUAAUA-5'; DsiRNA 2, 5'GUUACGUGUUCCA CAGAAUAAAATA-3' and 3'-UUCA AUGCACAAGGUGUCUUAUUUUAU-5'). A square wave protocol (500 V, 5 ms, 0 gap, 1 pulse) was used to electroporate the cells (Gene Pulser Xcell device, Bio-Rad Laboratories). Immediately after electroporation, cells were transferred to 5 mL of IMDM medium supplemented with 10% FBS (preheated at 37ºC) and incubated for 20 minutes at 37°C in 5% CO_2. Thereafter, cells were centrifuged and transferred to SCF medium. Five days after electroporation, a repeated analysis of expression and functionality of the PBCMCs was performed as described above. The period of 5 days was determined by performing a follow-up analysis of expression at different time points after electroporation. Transfection efficiency was examined by electroporating the cells with 1 µg of mRNA encoding enhanced green fluorescent protein per 10^6 cells. mRNA against EGFP was prepared as described elsewhere [26]. Briefly, SoloPack Golden supercompetent *Escherichia 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 was isolated and purified using the Nucleobond Xtra Midi EF and Nucleobond finalizer kits (Macherey-Nagel). The isolated DNAs were digested with Spe-I restriction enzyme (ThermoFisher Scientific). Capped mRNA transcripts were synthesized from linearized plasmids and purified by DNase digestion and LiCl precipitation using an mMessage mMachine T7 in vitro transcription kit (Life Technologies).

Microscopy Analyses

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

Statistical Analysis

GraphPad Prism version 7 software was used for data analysis, and paired *t* tests were performed. The results are expressed as mean (SEM). A P value of <.05 was considered significant. The "n" in the figures denotes the total number of donors used.

Results

PBCMCs were cultured from the peripheral blood of 17 healthy volunteers without drug hypersensitivity. Human PBCMCs were defined as viable cells expressing both CD117 and CD203c [15]. As shown in Figure 1 of the Online Repository, PBCMCs harbor 2 distinct subpopulations, namely, a subpopulation expressing MRGPRX2 ($MRGPRX2^+$) and a subpopulation negative for MRGPRX2 ($MRGPRX2^-$). Each culture yields $MRGPRX2^+$ cells with a mean expression of 52% ± 3% $MRGPRX2^+$ cells (n=17), thus enabling comparative analyses. Preliminary dose-response curve studies for the drugs evaluated were used to determine the optimal stimulation concentrations 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 Material and Methods.

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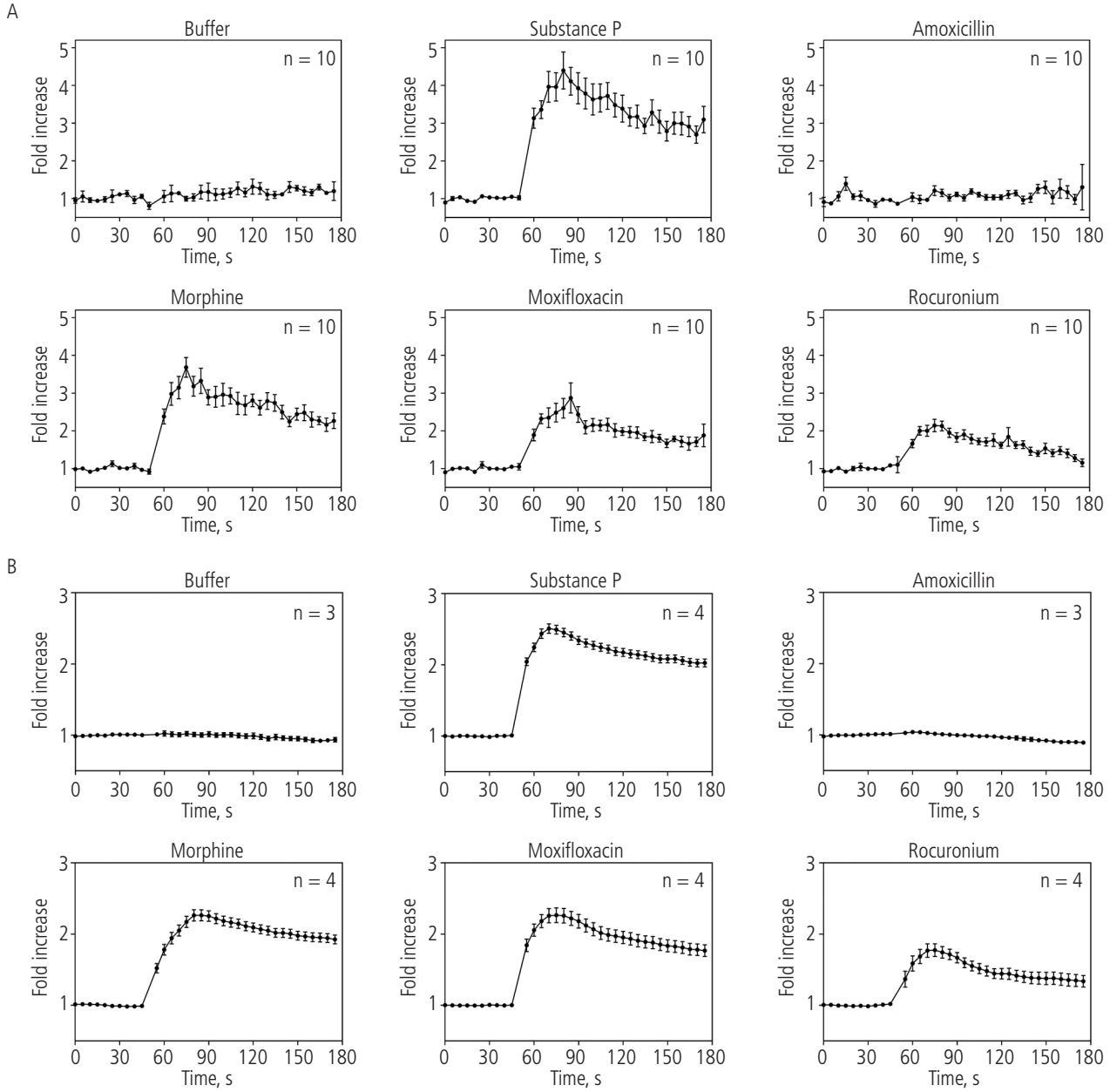

Figure 1. Calcium imaging of human PBCMCs. A, Flow cytometry data. B, Microscopy data. Incubation with buffer, the positive control substance P (74 μM), the natural agonist of MRGPRX2, amoxicillin (1370 μM), morphine (500 μM), moxifloxacin (571 μM), or rocuronium (1640 μM). Fold increase was calculated against the basal intensity. PBCMC indicates MCs cultured from CD34$^+$ progenitor cells obtained from peripheral blood; MRGPRX2, Mas-related G protein–coupled receptor X2.

The kinetics of changes in intracellular calcium levels after stimulation of PBCMCs is displayed in Figure 1, and representative plots are shown in Figure 3 and in Movies 1-4 of the Online Repository. Stimulation with substance P, morphine, and moxifloxacin resulted in a rapid increase in intracellular calcium, which peaks within approximately 75 seconds and slowly fades without returning to baseline after 3 minutes. Stimulation with rocuronium at a maximum nontoxic concentration enhanced intracellular calcium levels, although this elevation was less pronounced and almost returned to baseline levels after 3 minutes. Amoxicillin did 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 were restricted to the MRGPRX2$^+$ cells. In contrast, for substance P, calcium mobilization was also observed in MRGPRX2$^-$ cells and was less pronounced than in MRGPRX2$^+$ cells.

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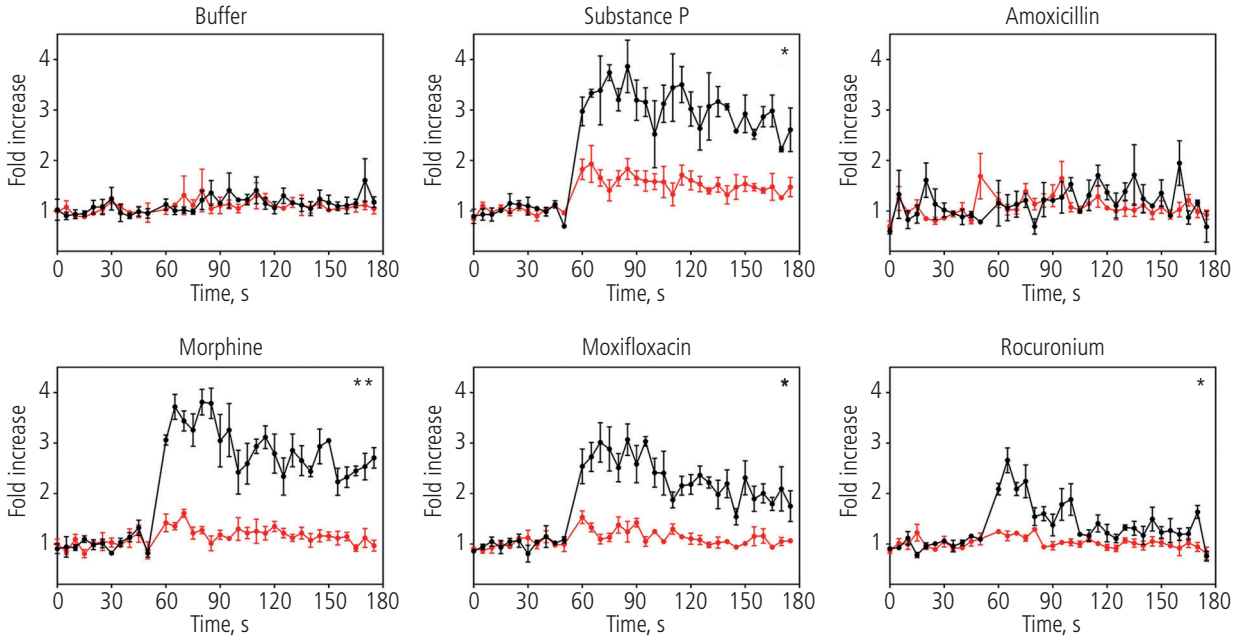

Figure 2. Flow cytometry–based calcium imaging in MRGPRX2+ (black) and MRGPRX2– (red) subpopulations. After 50 seconds, PBCMC samples were incubated with buffer, the natural ligand of MRGPRX2 substance P (74 μM), amoxicillin (1370 μM), morphine (500 μM), moxifloxacin (571 μM), or rocuronium (1640 μM). In all experiments, n=3. Areas under the curve were compared using a paired t test, $P<.05$*, $P<.01$**. Note that in the absence of MRGPRX2, there was no calcium response to the drugs, while there was a low response to the neurokinin substance P. MRGPRX2 indicates Mas-related G protein–coupled receptor X2; PBCMC, MCs cultured from CD34+ progenitor cells obtained from peripheral blood

Surface expression of the lysosomal degranulation marker CD63 is shown in Figure 3. No surface expression of CD63 was observed in cells stimulated with rocuronium or amoxicillin, likely reflecting insufficient or absent calcium mobilization, respectively. In contrast, CD63 was clearly upregulated when PBCMCs were incubated with substance P, morphine, or moxifloxacin (Movies 1-4). For the drugs, consistent with the findings of the calcium staining experiments, degranulation, as reflected by the upregulation of CD63, was restricted to MRGPRX2+ cells. Moreover, this upregulation was most pronounced in the cells expressing the highest baseline MRGPRX2 density on their plasma membrane. In contrast, substance P induced calcium mobilization in MRGPRX2+ and, to a lesser extent, in MRGPRX2– cells. Degranulation to the neurokinin was restricted to the MRGPRX2+ subpopulation, likely because of insufficient calcium mobilization in the MRGPRX2– cells (Figure 3B).

We confirmed the role of MRGPRX2 by applying a silencing technique using electroporation to enable the entrance of DsiRNA [18,26]. As shown in Figure 5 of the Online Repository, based on EGFP expression, this approach had a transfection efficiency in our PBCMCs of 60%-90% (n=3). In this knock-down model, de novo expression of MRGPRX2 was markedly downregulated, causing a reduction in surface levels of the receptor. In MRGPRX2-DsiRNA–treated cells, we observed a 77% ± 4% decrease in MRGPRX2 expression compared to cells electroporated with the control DsiRNA. The maximal expression of FcεRI remained unaltered in both conditions (Figure 6 of the Online Respository).

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

Discussion

Activation of MRGPRX2, which is constitutively expressed by MCs in human skin [13], constitutes 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 MCs obtained from peripheral blood CD34+ progenitor cells of healthy

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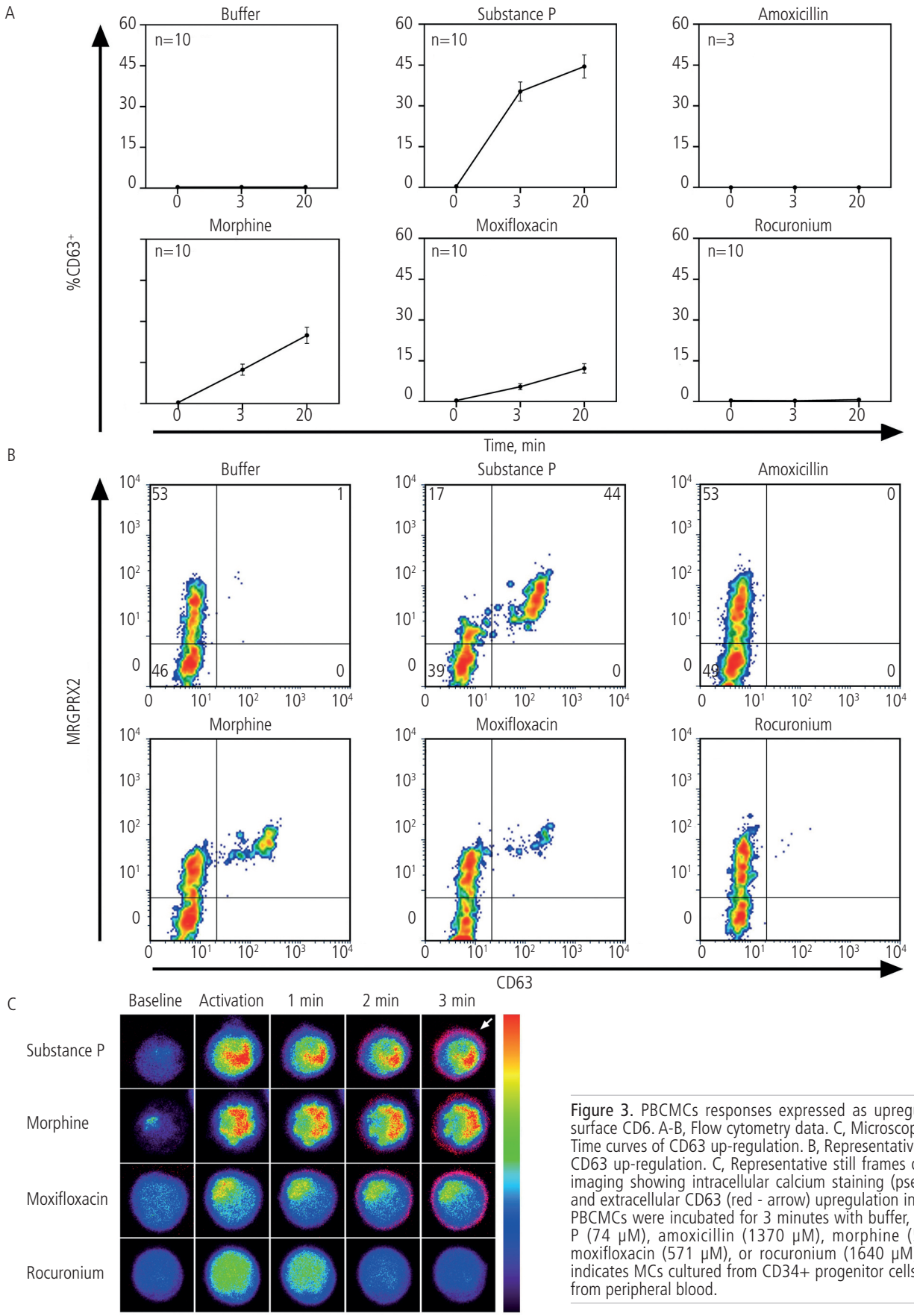

Figure 3. PBCMCs responses expressed as upregulation of surface CD6. A-B, Flow cytometry data. C, Microscopy data. A, Time curves of CD63 up-regulation. B, Representative plots for CD63 up-regulation. C, Representative still frames of live cell imaging showing intracellular calcium staining (pseudocolor) and extracellular CD63 (red - arrow) upregulation in PBCMCs. PBCMCs were incubated for 3 minutes with buffer, substance P (74 μM), amoxicillin (1370 μM), morphine (500 μM), moxifloxacin (571 μM), or rocuronium (1640 μM). PBCMC indicates MCs cultured from CD34+ progenitor cells obtained from peripheral blood.

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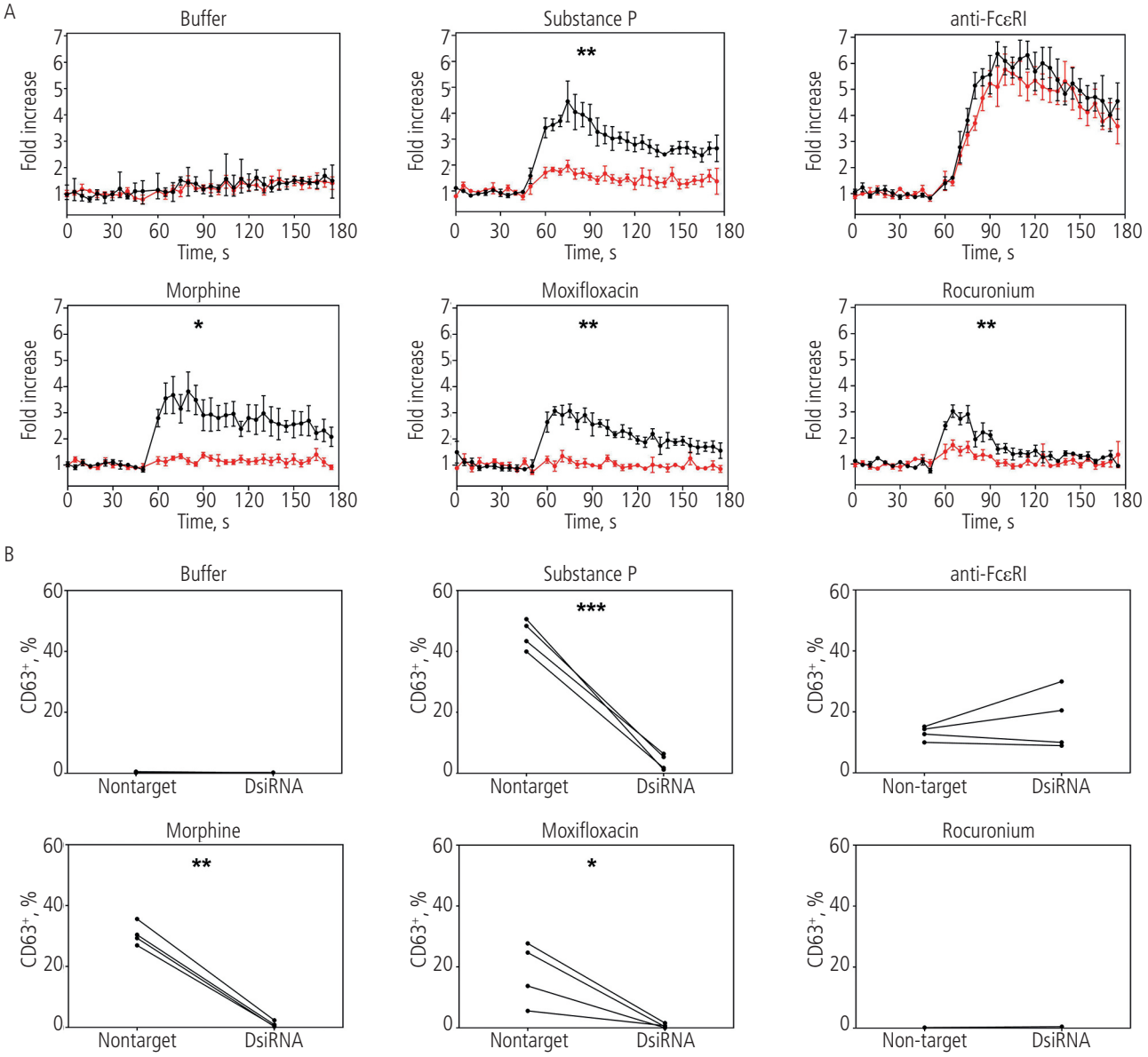

Figure 4. Effect of MRGPRX2 silencing on PBCMC functionality after MRGPRX2-specific DsiRNA electroporation. PBCMCs were electroporated with a negative control DsiRNA (black) or a MRGPRX2-specific DsiRNA (red). (A) Effect of the silencing on the calcium levels and (B) CD63 up-regulation after 3 minutes 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 *t* test, $P<.05$*, $P<.01$**, $P<.001$***. Note that the effect of MRGPRX2 silencing is consistent with the differences observed between MRGPRX2$^+$ and MRGPRX2$^-$ cells, as shown in Figures 2 and 3. MRGPRX2 indicates Mas-related G protein–coupled receptor X2; PBCMC, MCs cultured from CD34$^+$ progenitor cells obtained from peripheral blood.

donors [14,15] and used DsiRNA electroporation to further explore the potential of certain drugs to act as MRGPRX2-dependent MC secretagogues. Our results demonstrate that the combination of human MCs, DsiRNA electroporation–based downregulation of MRGPRX2, and multicolor 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, that is, detection of limited changes in protein expression 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 proof of concept that our approach can eliminate some of the uncertainties and deepen our mechanistic understanding of the MRGPRX2-related endotype of IDH. Importantly, we show that morphine, moxifloxacin, and rocuronium, but not amoxicillin, induce an MRGPRX2-dependent increase in intracellular calcium, which is accompanied by degranulation,

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expressed as CD63 upregulation, in the case of 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 that substance P can also trigger some MRGPRX2$^-$ 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 MCs [27,28] that are not suppressed by our silencing technique.

The dissociation between calcium signals and degranulation after stimulation with a maximum nontoxic concentration for rocuronium is intriguing. Although we cannot rule out the possibility that this phenomenon results from suboptimal 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 consistent with observations in other human cell line models, thus 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].

Fluoroquinolones can cause severe IDH reactions that are difficult, if not impossible, to document because of uncertainties associated with these nonspecific histamine releasers in skin testing [22,29,30], drug-specific IgE assays [31,32], and basophil activation tests [23,32-35]. The frequent occurrence of IDH to fluoroquinolones 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 an increase in intracellular calcium associated with degranulation of MRGPRX2$^+$ PBCMCs and that these processes are abrogated by selective silencing of MRGPRX2 via DsiRNA electroporation. The findings are in line with the observations reported by others [2,5], namely, that moxifloxacin (among other fluoroquinolones) potentially leads to IDH via occupancy of MRGPRX2. The earlier failure of LAD2 cells to reveal MRGPRX2 activation by moxifloxacin [3] could be associated with the variable differentiation status of this cell line, thus 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 specific for IgE/FcεRI–dependent reactions, whereas IgE/FcεRI–independent IDH to NMBAs have been thought to yield negative skin test responses. However, in the light of the observations by McNeil et al [2], it has been suggested that the value of skin testing should be verified and that anaphylaxis induced by NMBA should be reclassified [38]. In fact, the authors hypothesize that most NMBAs can cause non-IgE/FcεRI–mediated IDH rather than IgE/FcεRI–dependent IDH. However, our current observations argue against an excessively generalized mechanistic reclassification of NMBA-induced hypersensitivity reactions.

Rocuronium elicits a relatively limited and transient elevation of intracellular calcium in MRGPRX2$^+$ cells, although these changes are not associated with MC degranulation. This observation parallels the negative findings of Lansu et al [3] and Navinés-Ferrer et al [8], which were based on a human cell line model. The discrepancy between murine and human MCs related to Mrgprb2 and MRGPRX2, respectively, is best explained by adaptive changes in the *MRGPRX2* gene in human evolution [10], which render 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), the basophil activation test, and skin testing in the vast majority of patients, indicating that rocuronium-induced anaphylaxis results predominantly from IgE/FcεRI cross-linking [21]. Admittedly, most patients who experience rocuronium-induced anaphylaxis are also drug-naïve, and prior sensitization in this group is likely to result from cross-reactivity towards household and other environmental agents and medicines [39] that harbor structurally similar epitopes, especially tertiary and quaternary substituted ammonium structures [40] and compounds that might trigger excessive non–allergen-specific IgE production such as pholcodine [41]. Of note, the finding that calcium staining was not associated with upregulation of CD63 calls for further investigation with other lysosome-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], most IDH reactions to opiates result from nonspecific mediator release. The correct diagnosis of rare IgE/FcεRI–dependent IDH to these drugs is difficult, mainly because of the absence of reliable drug-specific IgE assays [25] and uncertainties associated with nonspecific skin testing [43]. Although the first description of morphine to activate MRGPRX2 dates back to 2007 [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 leads to elevated intracellular calcium levels and degranulation of human MRGPRX2$^+$ MCs, which can be inhibited by selective silencing of the receptor. Our data also explain why skin tests are not appropriate for distinguishing 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 tests can help to correctly diagnose IgE/FcεRI opiate hypersensitivity and to prevent erroneous overdiagnosis [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 β-lactam antibiotic. However, in line with earlier observations on proteinaceous allergens [15] and our recent experience with chlorhexidine [48], it is attractive to speculate that MCs might become responsive to this β-lactam antibiotic after passive sensitization with sera from patients with IgE/FcεRI–dependent amoxicillin allergy.

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Although our experiments add to knowledge of MRGPRX2-dependent drug hypersensitivity, the observation that only a minority of the patients experience such reactions and that these reactions are generally restricted to a specific drug (or drug class) remains a conundrum. As elegantly addressed by Porebski et al [6], one could hypothesize that these observations are related to genetic polymorphisms and mutations resulting in augmented responsiveness of the receptor, specific receptor-binding sites, differences in the MRGPRX2 signalosome, epigenetic modifications, posttranscriptional modifications resulting in synthesis of MRGPRX2 variants, temporarily or constitutively varying surface expressions, and even in the influence of cofactors. In this context, a limitation of our method, as in many in vitro and ex vivo experiments, lies in the need for supratherapeutic stimulation concentrations (sometimes near toxic, as seen for rocuronium) that might blunt subtle differences in affinity or avidity of the molecules for MRGPRX2. Direct comparisons between compounds harboring different numbers of tetrahydroisoquinolines may, to some extent, shed light on this issue.

Taken together, comparative analyses of human MRGPRX2^{+} and MRGPRX2^{-} MCs and selective silencing of MRGPRX2 via DsiRNA electroporation coupled to flow cytometry can clarify uncertainties and improve our understanding of the MRGPRX2 pathway in IDH. Our data advise prudent interpretation of changes in intracellular calcium in the field of IDH, as induction of calcium signals does not necessarily translate into degranulation. The use of this approach may eventually call for the reclassification of IDH in reactions such as IgE/FcεRI–dependent and MRGPRX2-dependent reactions (with largely unknown mechanisms). 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 [1], and to compare this approach with other means of identifying human MC degranulators [17]. The use of our model to improve our understanding of the pathogenic mechanisms of IDH could culminate in the development of novel diagnostic and therapeutic options, including MRGPRX2 antagonists, for the prevention and treatment of IDH.

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Conflicts of Interest

M Maurer has received honoraria (advisory board, speaker) and/or institutional grant/research support from Allakos, Amgen, AstraZeneca, Bayer, Dr. Pfleger, FAES, Genentech, GSK, Innate Pharma, Kyowa Kirin, Lilly, Merckle Recordati, Moxie, Novartis, Regeneron, Roche, Sanofi, MSD, UCB, and Uriach. The remaining authors declare that they have no conflicts of interest.

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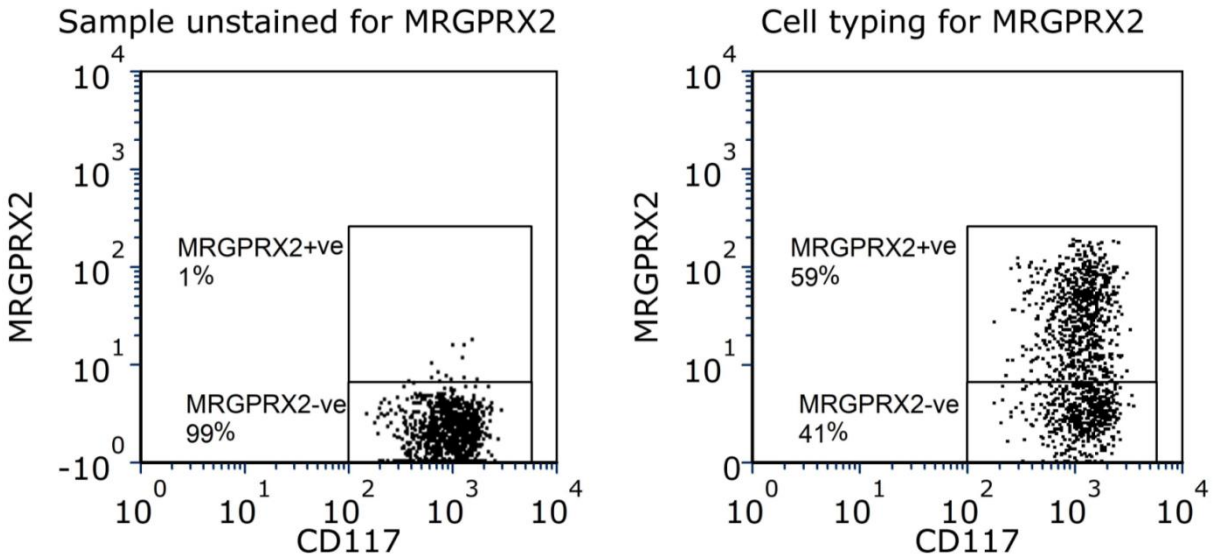
SUPPLEMENTARY MATERIAL

Figure 1. Representative plot for the MRGPRX2 expression on PBCMCs.



Peripheral blood cultured mast cells (PBCMCs) are defined as $CD117^{+ve}CD203c^{+ve}$ cells. PBCMCs harbour two subpopulations: cells with surface expression ofMRGPRX2 ($MRGPRX2^{+ve}$) and cells without expression ofMRGPRX2 ($MRGPRX2^{-ve}$).

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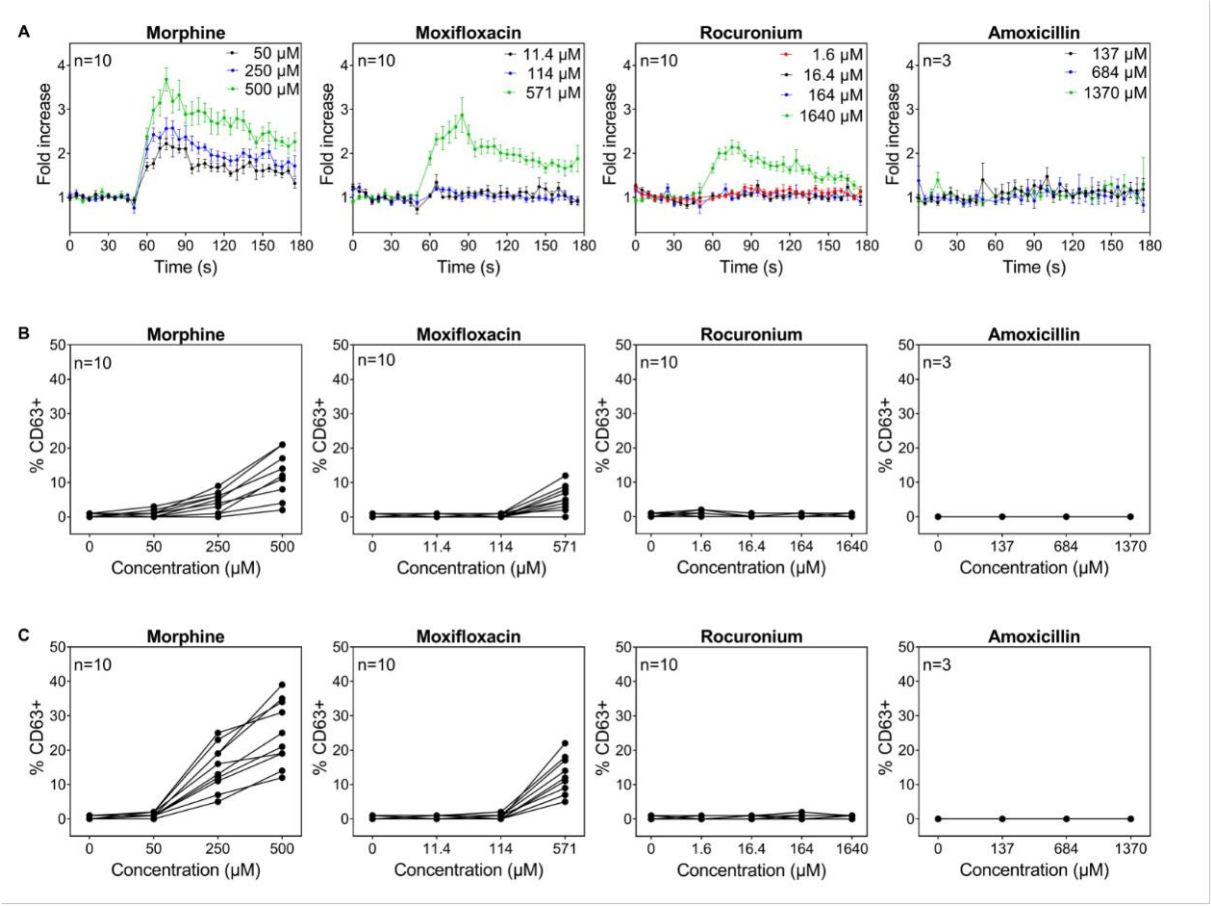
Figure 2. Dose-response curves for changes in intracellular calcium and CD63expressionin PBCMCs.



(A) Dose-response curves of intracellular calcium levels. **(B)** Dose-response curves of CD63 up-regulation after 3 min of stimulation or **(C)** after 20 min of stimulation. Attempts to increase the rocuronium concentration revealed to be cytotoxic.

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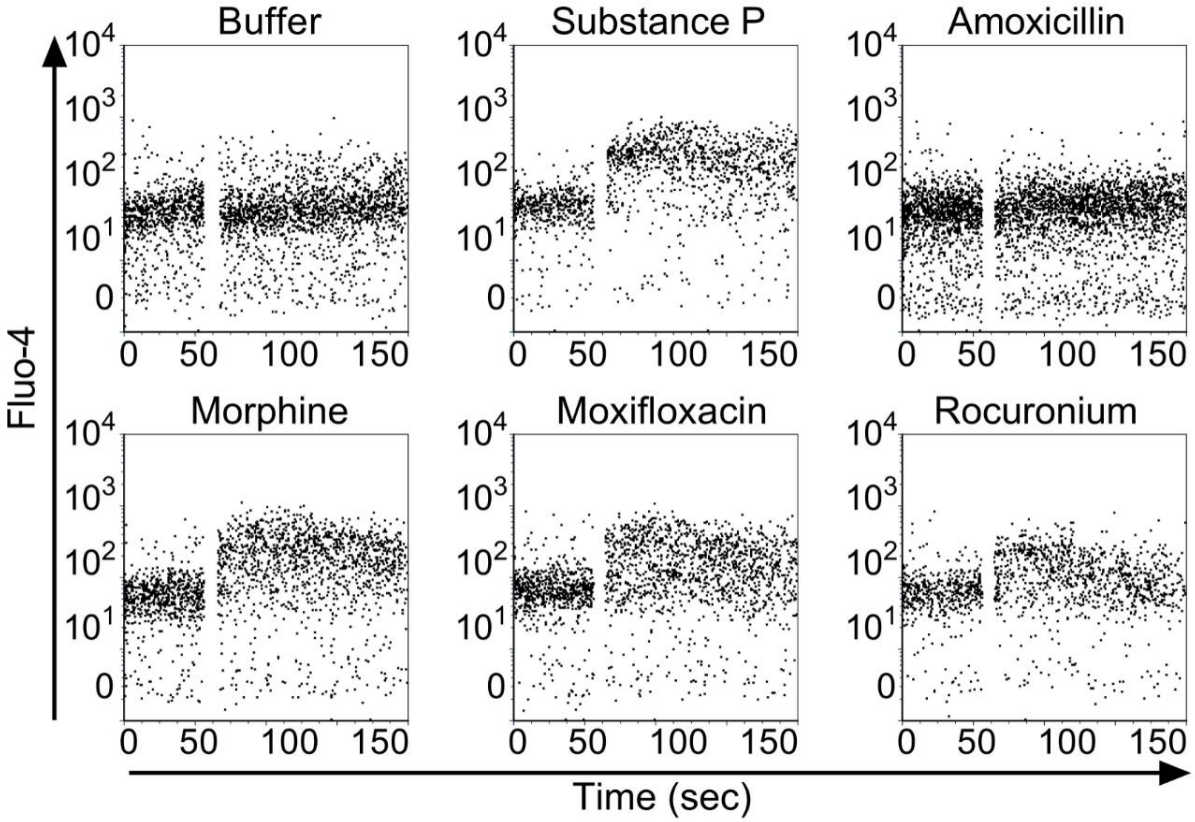
Figure 3. Representative plots for intracellular calcium imaging in PBCMC.



PBCMCs were, after 50 sec, stimulated with buffer, substance P (74 µM), amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM).

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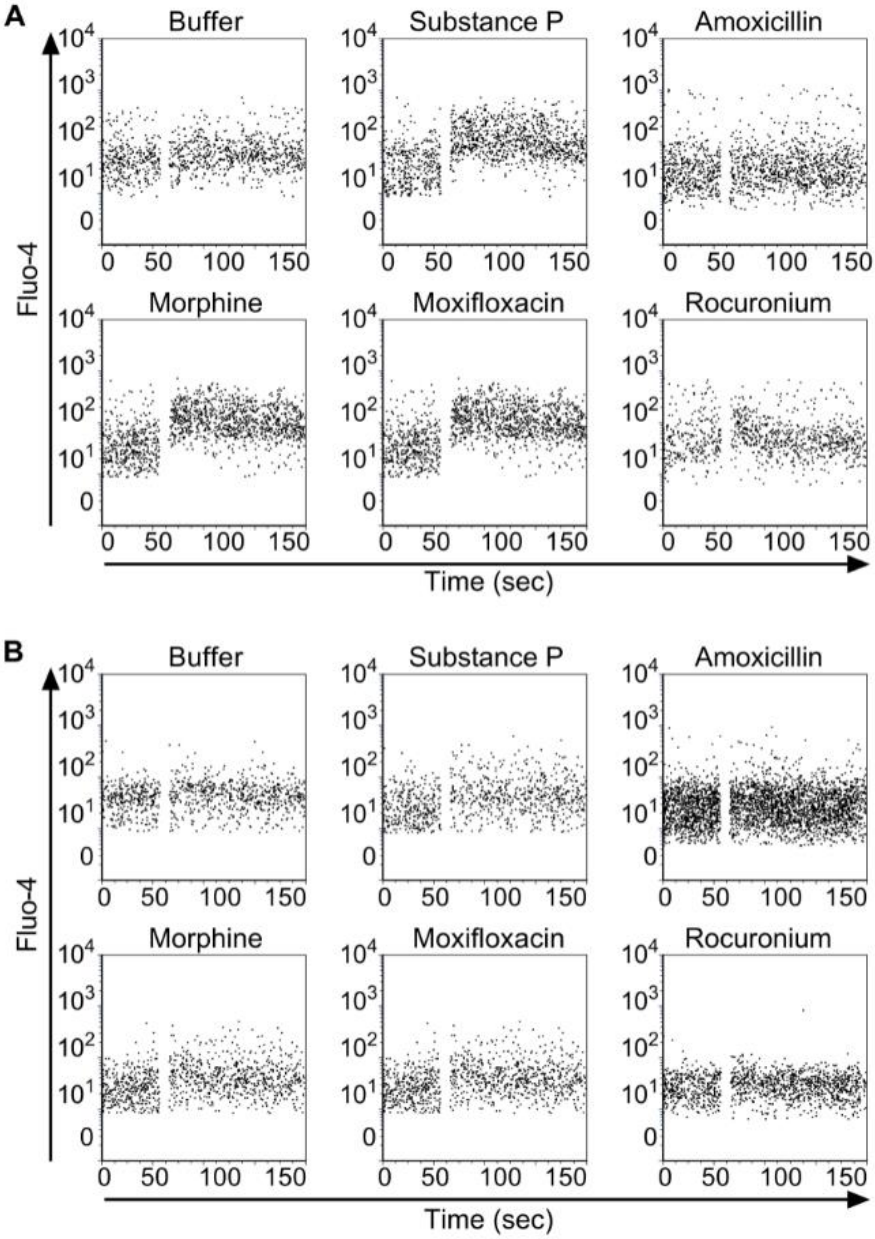
Figure 4. Representative plot for intracellular calcium imaging in MRGPRX2^{+ve} (A) and MRGPRX2^{-ve} (B) subpopulations.



PBCMC samples were (after 50 sec) incubated with buffer, the natural ligand of MRGPRX2 substance P (74 µM), amoxicillin (1370 µM), morphine (500 µM), moxifloxacin (571 µM) or rocuronium (1640 µM).

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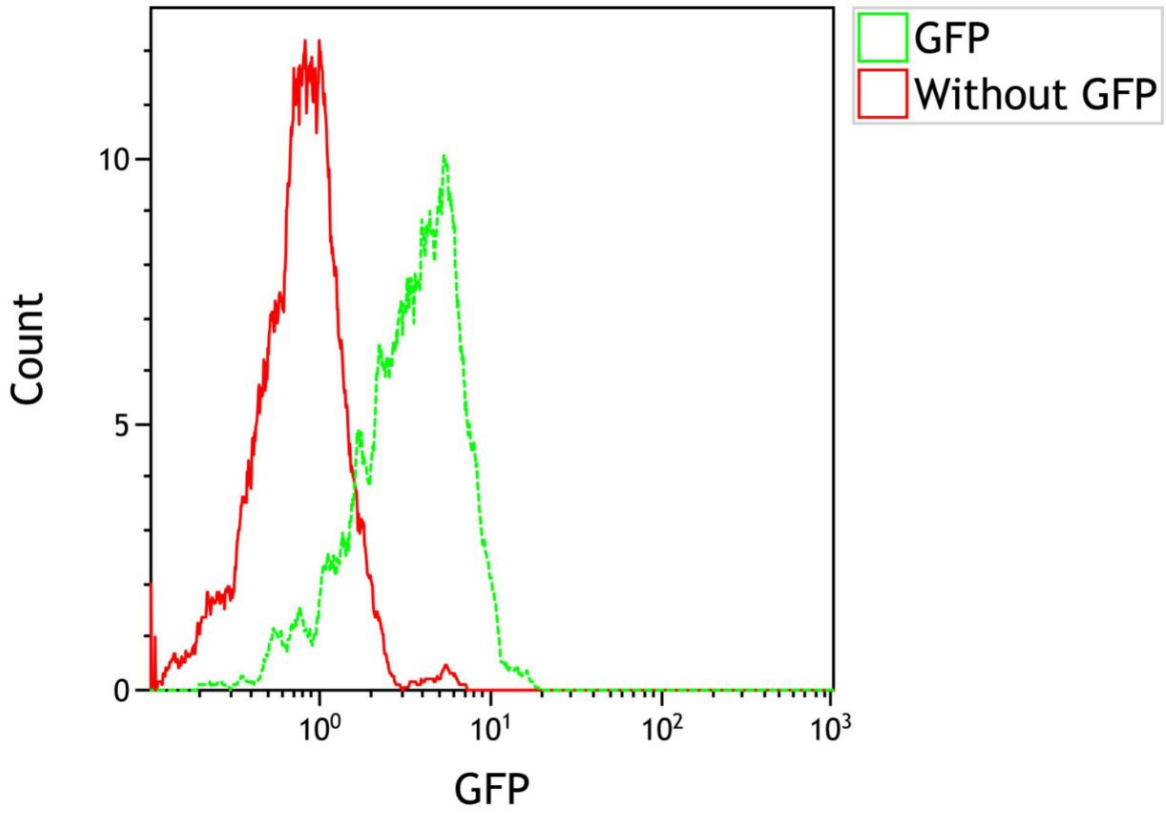
Figure 5. Representative plot of the transfection efficiency using enhanced green fluorescent protein (EGFP) mRNA.



PBCMCs areelectroporated with (green dot line) or without (red full line) EGFP mRNA as a surrogate for transfection efficiency of RNA electroporation. 24h after EGFP expression is analyzed using flow cytometry.

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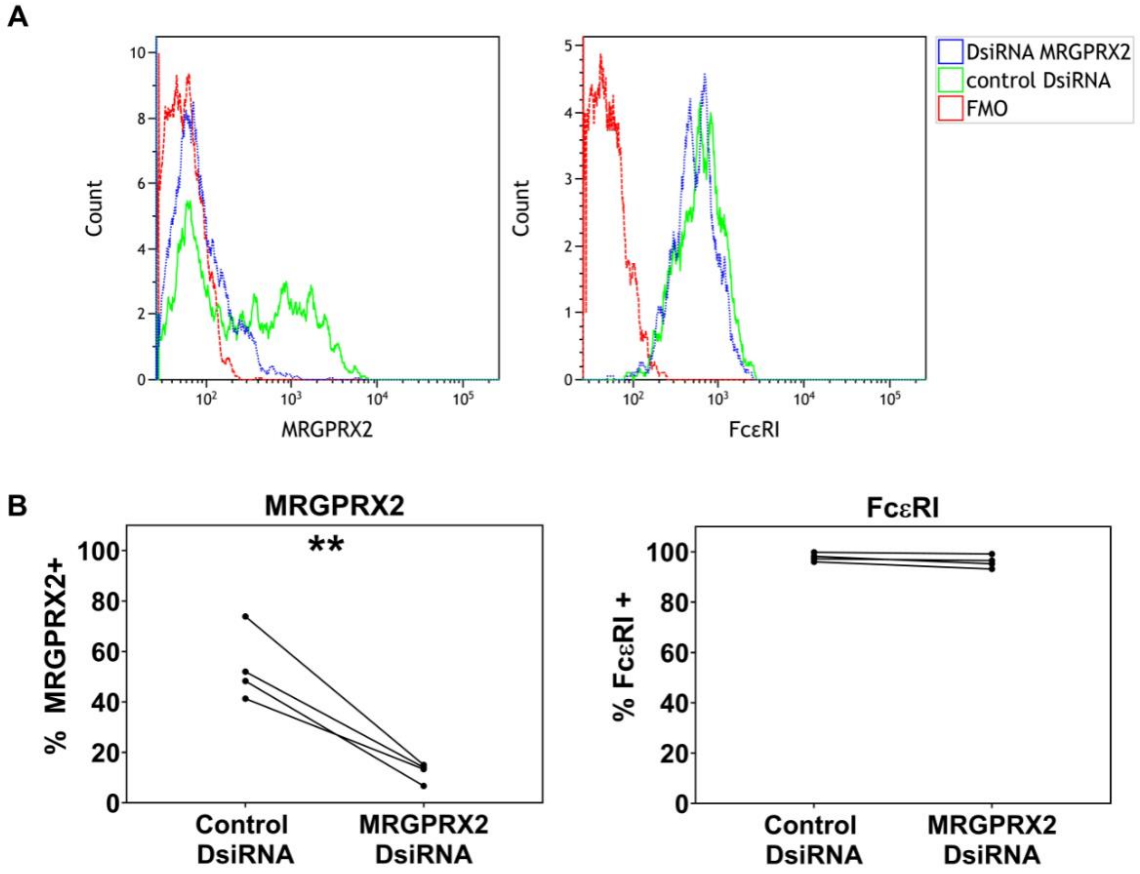
Figure 6. Silencing of the MRGPRX2-receptor after electroporation with anti-MRGPRX2 DsiRNA.



(A) Representative plots or **(B)** a comparison of the surface expression of MRGPRX2orFcεRI between PBCMC electroporated with non-targeting DsiRNAorDsiRNA specific for MRGPRX2.In all experiments, n=4. $p < 0.01$**.

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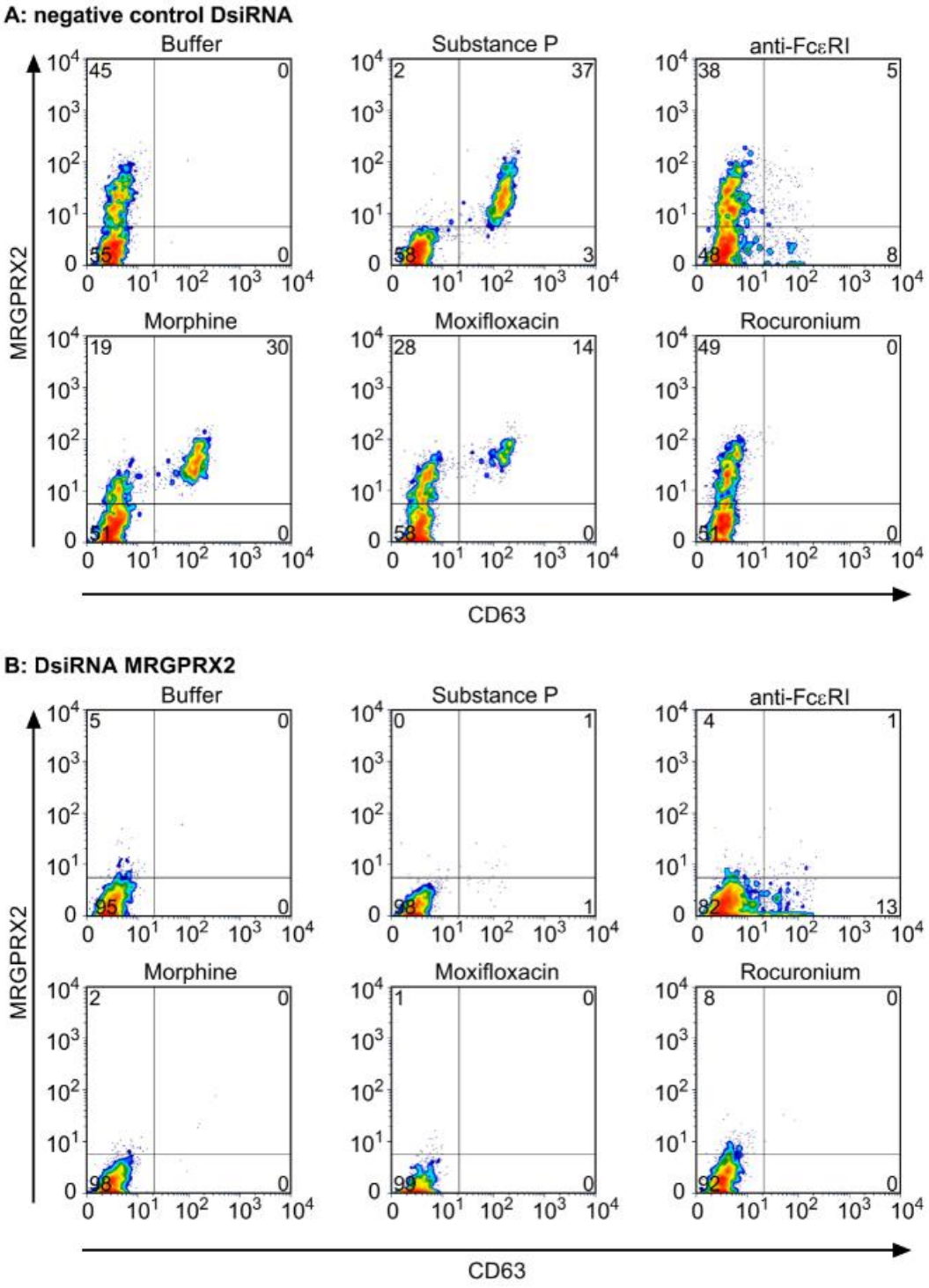
Figure 7. Representative plots of CD63 up-regulation after silencing of MRGPRX2.



Cells were electroporated with a negative control**(A)** or target specific DsiRNA**(B)**. Thereafter, cells were incubated with buffer, substance P (74 µM), anti-FcεRI(2.5 µg/mL), morphine (500 µM), moxifloxacin (571 µM)orrocuronium (1640 µM).

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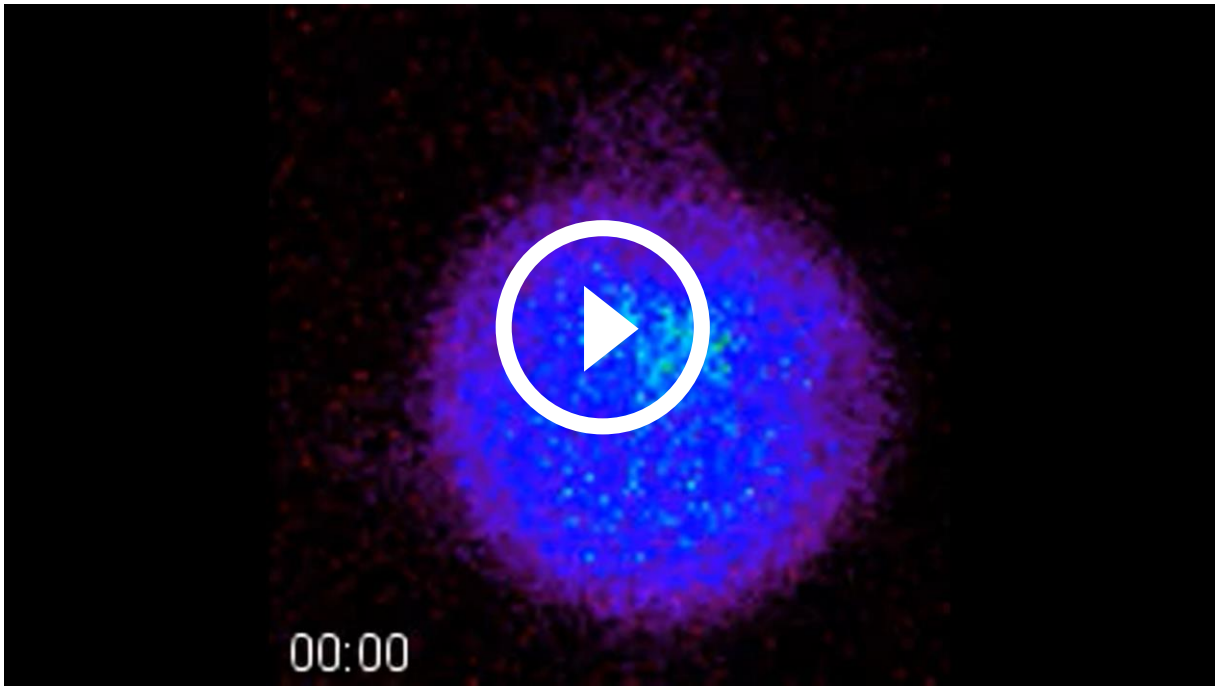
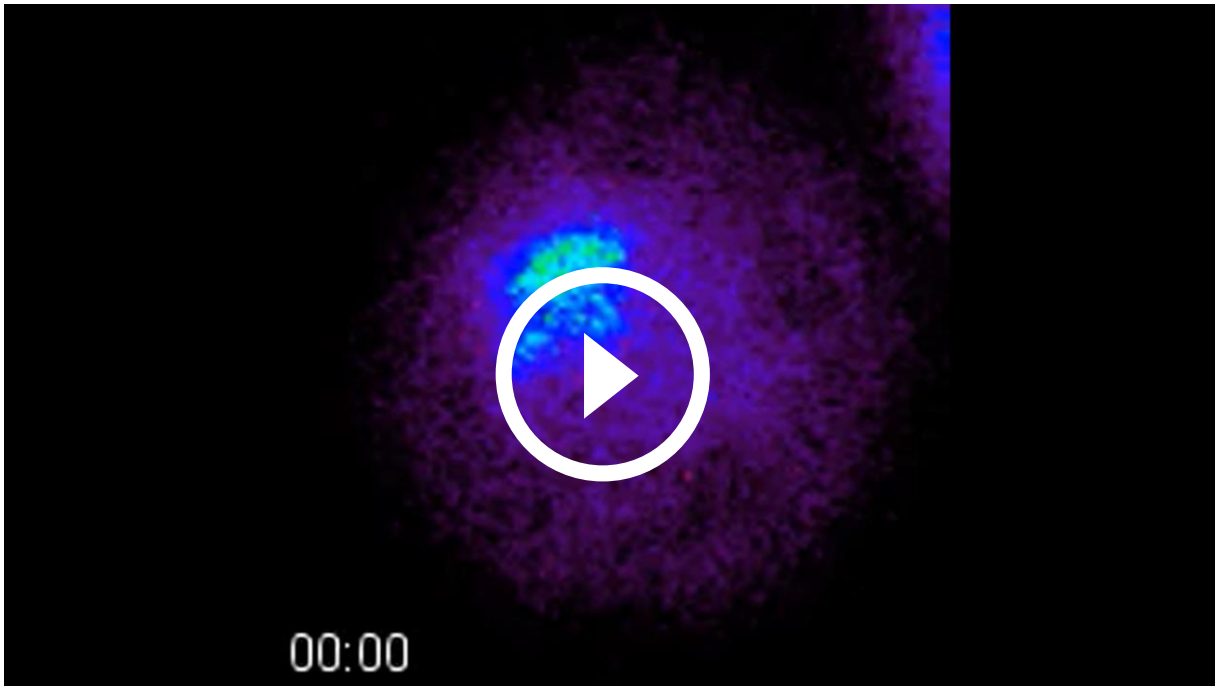
Movie 1: Substance P-stimulated PBCMC (74 µM)



Movie 2: Morphine-stimulated PBCMC (500 µM)



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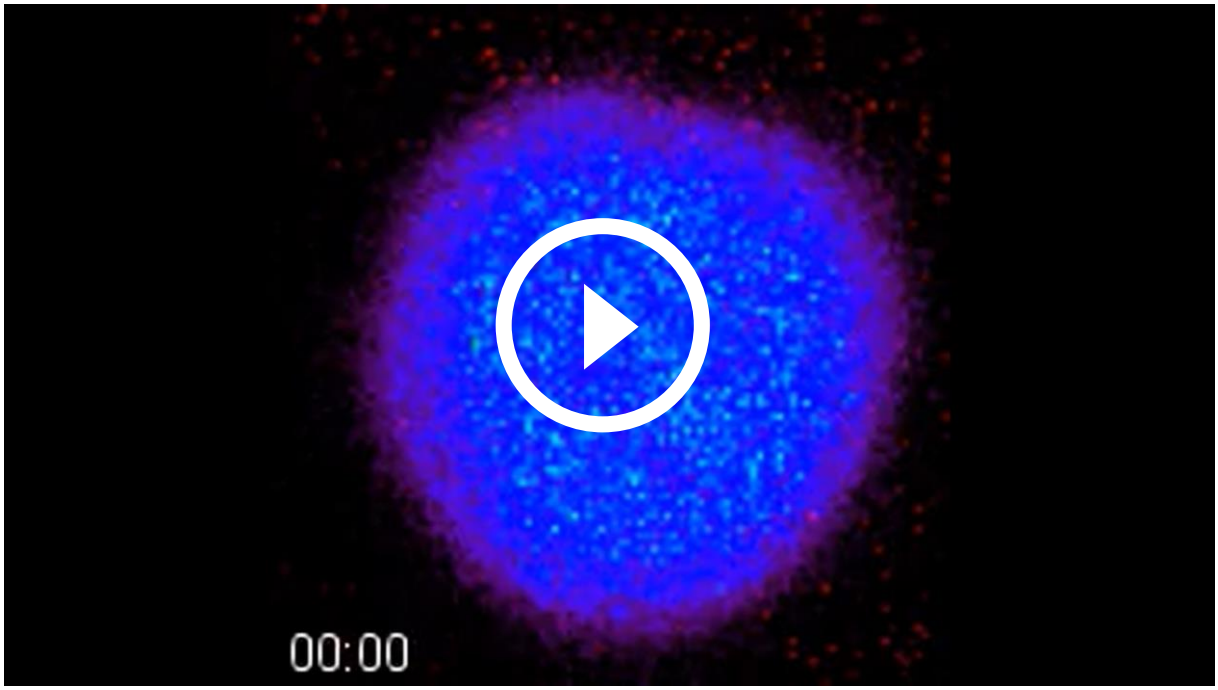
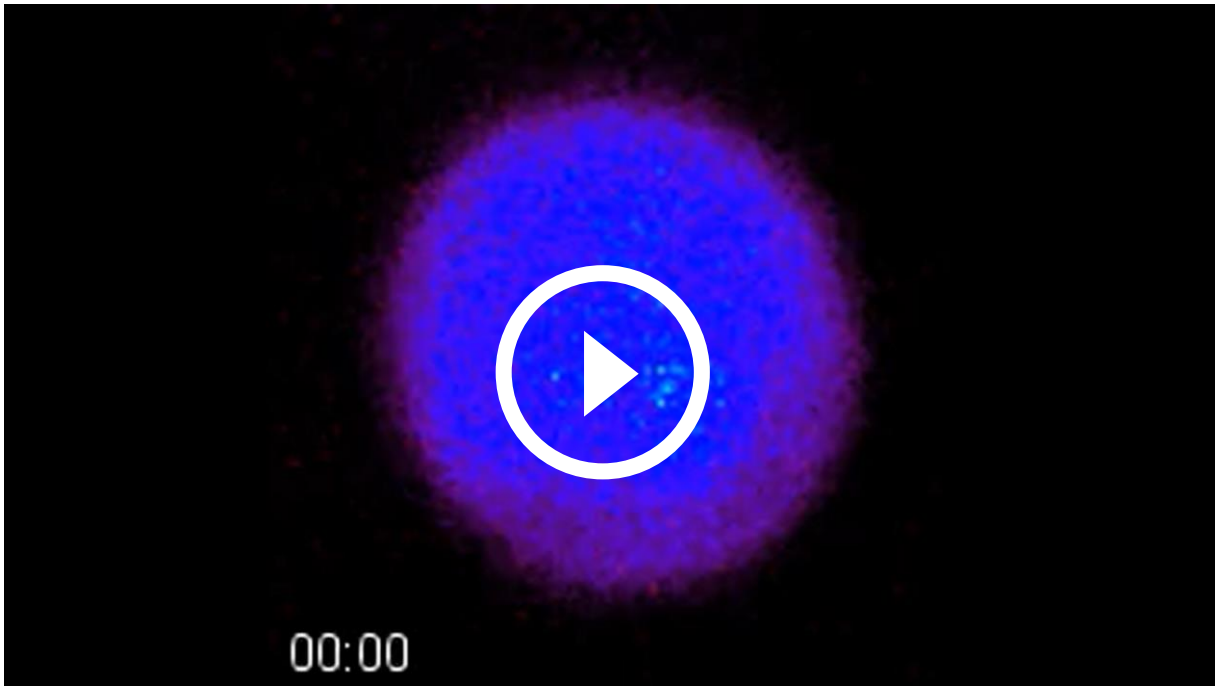
Movie 3: Moxifloxacin-stimulated PBCMC (571 µM)



Movie 4: Rocuronium-stimulated PBCMC (1640 µM)



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