

Mivacurium induce mast cell activation and pseudo-allergic reactions via MAS-related G protein coupled receptor-X2

Delu Che^a, Jue Wang^a, Yuanyuan Ding^a, Rui Liu^a, Jiao Cao^a, Yongjing Zhang^a, Yajing Hou^a, Hongli An^b, Zijun Gao^{c,*}, Tao Zhang^{a,*}

^a College of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China

^b Center for Translational Medicine, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China

^c Department of Anesthesiology, Xi'an Honghui Hospital of Xi'an Jiaotong University, Xi'an 710054, China

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ABSTRACT

Background: Mivacurium is a non-depolarizing muscle relaxant and widely used as a short-acting anesthetic. Pseudo-allergic reactions to mivacurium occur when it is administered during perioperative anesthesia. These reactions may present a serious threat to the patient's life, particularly in children.

Methods: MAS-related G protein coupled receptor-related pseudo-allergic reactions that were induced by mivacurium were investigated using skin swelling and extravasation assays *in vivo* and mast cell degranulation assay *in vitro*.

Results: Mivacurium caused pseudo-allergic reactions in wild-type mice by inducing mast cells to release histamine. However, it did not induce a similar phenomenon in Kit^{W-sh/W-sh} mice. Furthermore, MrgprB2-knockout mice displayed no inflammatory response to mivacurium. Mivacurium induced LAD2 cell degranulation in a dose-dependent manner. Mivacurium stimulated intracellular calcium ion (Ca²⁺) influx in MRGPRX2-HEK293 cells but not in NC-HEK293 cells. However, mivacurium induced the release of only low levels of mediators in LAD2 cells transfected with MRGPRX2-targeted small interfering (si)RNA. Notably, cytokine release was not observed in LAD2 cells even when stimulated with high concentrations of mivacurium.

Conclusion: Mivacurium activated MRGPRX2 and triggered mast cell degranulation, leading to anaphylactoid reactions. However, mivacurium did not induce the release of other cytokines. Therefore, the targeting of MRGPRX2 can potentially block mivacurium-induced adverse drug effects, particularly pseudo-allergic reactions.

1. Introduction

Mivacurium is a benzyloisoquinoline derivative that is the shortest acting nondepolarizing muscle relaxant currently available [1]. Mivacurium is rapidly hydrolyzed in the plasma and has a short duration of action (< 10 min) [2]. Compared with rocuronium, mivacurium has many advantages [3,4], such as a rapid effect, non-neurological toxicity [5] and a lack of heart rate alteration [6]. Therefore, mivacurium is widely used for short-duration surgical procedures [7,8], including laparoscopic surgery [9] and tracheal cannulation [10], and in adults with major burns [11]. Because the recovery time from mivacurium in children is approximately 30% faster than that in adults [12], mivacurium is a particularly important muscle relaxant that is currently used in infants and young children [13,14].

However, mivacurium is not completely safe for use during the

perioperative period [15]. The most pronounced side effect of mivacurium is histamine release [16]. A mivacurium-induced anaphylactic reaction increases the surgical risk to patients [17,18]. Additionally, skin test results have shown that mivacurium causes cutaneous anaphylaxis at a low dosage [19]. The mechanism of mivacurium-induced anaphylactic reactions remains unclear. Currently, promethazine and dexamethasone are often used in the clinic for the pretreatment of mivacurium-induced histamine release in children [19]. However, this treatment is palliative.

Mast cells or basophils are the key effectors of pseudo-allergic reactions. McNeil et al discovered that MAS-related G protein coupled receptor (MRGPRX2) on mast cells is the target that directly induces pseudo-allergic reactions and verified that fluoroquinolones and neuromuscular relaxants can cause anaphylactoid reactions by acting on MRGPRX2, leading to the degranulation of mast cells [20].

* Corresponding authors at: School of Pharmacy, Xi'an Jiaotong University, Yanta Westroad, Xi'an 710061, China (T. Zhang).

E-mail addresses: kobe84629@163.com (Z. Gao), taozhang@mail.xjtu.edu.cn (T. Zhang).

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Subramanian H et al subsequently confirmed that the MRGPRX2 receptor acts as an intermediary agent of anaphylactoid reactions [21].

To ensure the safe use of mivacurium, this study was conducted to investigate whether mivacurium induces pseudo-allergic reactions and to clarify the mechanism of mivacurium-induced pseudo-allergic reactions mediated through mast cell-related MRGPRX2.

2. Materials and methods

2.1. Drugs and reagents

Compound 48/80 was purchased from Sigma-Aldrich (St. Louis, USA). Mivacurium was obtained from Meilunbio Co., Ltd. (Dalian, China) and was purified to $\geq 98\%$. Fluo-3, AM ester and Pluronic F-127 were obtained from Biotium (California, USA). TM buffer was prepared on the day of experiments (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l CaCl₂, 0.143 g/l MgSO₄, 0.162 g/l KH₂PO₄, 2.383 g/l HEPES, 0.991 g/l glucose and 1 g/l BSA, pH = 7). The p-nitrophenyl N-acetyl- β -D-glucosamide and Triton X-100 reagents were procured from Sigma Aldrich Co., LLC. (St. Louis, USA) and were prepared at the proper concentrations prior to use. The stop buffer consisted of 0.1 M sodium carbonate and sodium bicarbonate, pH = 11. All aqueous solutions were prepared using ultrapure water produced by an MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Mouse model

C57BL/6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Adult male mice aged 8 weeks were used in the study. C57BL/6 MrgprB2-knockout mice were provided as a kind gift from the laboratory of Xinzhong Dong at Johns Hopkins University. C57BL/6 STOCK Kit^{W-sh}/HNihrJaeBsmJNju mice were purchased from the Model Animal Research Center of Nanjing University. All the mice were housed in the Experimental Animal Center of Xi'an Jiaotong University. The mice were housed in individual cages in a large colony room with free access to water and were fed a standard dry food twice per day. The breeding environment was 20–25 °C, with a relative humidity of 40% and a day-night cycle of 12/12 h. The mice were randomly divided into the following groups (5 mice per group): control, compound 48/80, and the experimental group. No randomization was used for the MrgprB2-knockout mice or the STOCK Kit^{W-sh}/HNihrJaeBsmJNju mice because randomization was not applicable to these studies. All animal experiments involving equal treatments were conducted by experimenters who were blinded to the conditions.

2.3. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols involving mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were under pentobarbital sodium anesthesia when undergoing any operation.

2.4. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). The cells were maintained in StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mM L-glutamine and 100 ng/ml human stem cell factor in an atmosphere containing 5% CO₂ at 37 °C. The culture medium was replaced every 2 weeks, and the cells were maintained at a density of 2×10^6 cells/ml.

Human MRGPRX2-expressing HEK293 cells were constructed by HIV-1-based lentiviral vectors and cultured by DMEM.

2.5. Peritoneal mast cell purification

Adult male C57BL/6 mice, 8 weeks of age, were sacrificed through CO₂ inhalation. A total of 12 ml of ice-cold mast cell dissociation medium (15 ml of MCDM: 1.5 ml of 10 × HBSS, 450 μ l of fetal bovine serum, 150 μ l of 1 M HEPES, 12.9 ml of sterile water, pH = 7.2) was used for two to three sequential peritoneal lavages, in which the MCDM and cells were mixed and then centrifuged at 200g for 10 min at 4 °C. The cells from each mouse were resuspended in 2 ml of MCDM and then layered over 4 ml of an isotonic 70% Percoll suspension (2.8 ml of Percoll, 320 μ l of 10 × HBSS, 40 μ l of 1 M HEPES, 830 μ l of MCDM) and centrifuged at 500g for 20 min at 4 °C. The mast cells in the pellet were resuspended, transferred to a new centrifuge tube and then diluted with MCDM at twice the volume of the suspension. This solution was mixed well and then centrifuged at 200g for 10 min at 4 °C. The mast cells in the pellet were retrieved, and their purity was > 95% as assayed by morphology. The mast cells were then resuspended in DMEM with 100 ng/ml recombinant mouse stem cell factor (SCF) and seeded into 96-well plates.

2.6. Hind paw swelling and extravasation

Adult male C57BL/6 mice, 8 weeks of age were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) pentobarbital sodium. Fifteen minutes after the induction of anesthesia, each mouse was injected intravenously (i.v.) with 0.2 ml of 0.4% Evans blue dye (EBD) in saline. A Vernier caliper was used to measure the thickness of the paw before any injection. Five minutes later, 5 μ l of the test drug was administered using a microinjector into the left paw, and saline was administered into the right paw as a negative control. Fifteen minutes later, the paw thickness was measured again and recorded. The mice were then sacrificed by decapitation, and a photo of each paw was obtained. The paw tissues were collected, dried for 24 h at 50 °C, and weighed. EBD was extracted by incubating 24 h in 500 μ l of acetone-saline (7:3) at 37 °C. The tissue samples were cut into pieces and placed for 10 min in an ultrasonic machine, followed by centrifugation for 20 min at 3000 rpm. The supernatant was equally distributed in 200- μ l aliquots into 96-well cell culture plates, and the OD was read at 620 nm using a spectrophotometer. For studies using drug substances, the mice were injected with 5 μ l of a 30 μ g/ml solution of compound 48/80 as a positive control.

2.7. Body temperature

Adult male mice weighing 10–22 g were anesthetized with an intraperitoneal injection of 0.15 ml of 0.35% pentobarbital sodium. The mice were injected i.v. with a saline solution of mivacurium (n = 6). Temperature transducers and a BL-410 biological function system (Chengdu Techman Software Co., Ltd., Chengdu, China) were then used for the analysis.

2.8. Skin allergy reaction and HE staining

Adult male mice weighing 18–22 g were immobilized on a board. The hair on the back was then depilated. The mice were intracutaneously injected in the shoulder with 50 μ l of a 1 mg/ml solution of mivacurium; a saline solution was used as a negative control. After 15 min, the injection location was removed from the skin and fixed in a concentration of 4% formaldehyde for 48 h. HE staining was performed according to standard procedures.

2.9. β -Hexosaminidase assay

The β -hexosaminidase assay was conducted in a 96-well plate. LAD2 cells were incubated overnight at 37 °C with 5% CO₂. Following incubation, the culture medium was removed, the drug substances were added at the indicated concentrations, and the cells were incubated for 30 min under 37 °C with 5% CO₂. The 96-well plate was then centrifuged at 200g for 5 min at 4 °C. To determine the total β -hexosaminidase content, the cells were lysed with 0.1% Triton X-100 in TM buffer. The β -hexosaminidase released into the supernatants and in the cell lysates was quantified by the hydrolysis of p-nitrophenyl N-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH = 4.5) for 90 min at 37 °C. The reaction was stopped by the addition of stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH = 11). The percentage of β -hexosaminidase release was assessed by measuring the absorption of the samples at 405 nm using a microplate spectrophotometer and was calculated as follows: absorbance of culture supernatant at 405 nm $\times$ 100/absorbance of total cell lysate supernatant at 405 nm. Compound 48/80 was used as a positive control.

2.10. Histamine release assay

In 96-well plates, 1×10^6 LAD2 cells were incubated overnight at 37 °C with 5% CO₂. Following incubation, the culture medium was removed, the drug substances were added at the indicated concentrations, and the cells were incubated for 30 min at 37 °C with 5% CO₂. The 96-well plate was centrifuged at 200g for 5 min at 4 °C. Histamine (HA) was purchased from Sigma, while histamine-2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), and HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Mass spectrometry-grade formic acid was obtained from Sigma. An LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used applying the LC-ESI-MS/MS method. Histamine was evaluated with the system by employing an HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μ m, Agela Technologies, Tianjin, China) and an isocratic elution with acetonitrile-water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

2.11. Chemokine release assay

LAD2 cells, 1×10^6 cells per well, were incubated in a 96-well plate overnight at 37 °C with 5% CO₂. The culture medium was removed, the drug substances were added at the indicated concentrations, and the cells were incubated for 12 h at 37 °C with 5% CO₂. The Human Chemokine Array Kit was purchased from R&D Systems China Co., Ltd. (Shanghai, China) and ELISA kits were purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China). All steps were performed strictly according to the manufacturers' instructions.

2.12. Intracellular calcium ion (Ca²⁺) mobilization assay

All drug substances were diluted to the required concentrations in calcium imaging buffer (CIB: 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO₃ and 20 mM sucrose, with the pH adjusted to 7.4 using NaOH). The incubation buffer consisted of 0.5 μ l of Fluo-3, 2 μ l of Pluronic F-127 and 997.5 μ l of CIB. MRGPRX2-HEK293 cells or NC-HEK293 cells were plated at 1×10^4 cells per well in a 96-well plate and incubated overnight at 37 °C with 5% CO₂. The cells were then washed twice in 100 μ l of CIB. Next, 100 μ l of incubation buffer was added, and the plate was incubated for 30 min at 37 °C with 5% CO₂. Then, the cells were again washed twice with CIB. The cells were used immediately for imaging. For the calcium imaging, the cells were magnified 200 times, and one photo per second was obtained under blue light. Cells were identified as responding if the [Ca²⁺]_i rose by at least 50% after the

substances were injected.

2.13. Small interfering (si)RNA transfection of LAD2 cells

Specific knockdown was achieved using small interfering (si)RNAs targeting MRGPRX2, and non-targeting siRNAs as negative control (NC). A SMART pool of double-stranded siRNAs targeting MrgprX2 and nonspecific siRNAs were obtained from Shanghai GenePharma Co., Ltd. The siRNA sequences were as follows: forward, 5'-GUACAACAGUGAAUGGAAATT-3' and reverse, 5'-UUUCCAUUCACUGUUGUACTT-3' for MRGPRX2; and forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3' for the control. For transfection, siRNA was delivered at a final concentration of 80 nM using the Lipofectamine® 2000 reagent according to the manufacturer's instructions. The cells were incubated for 48 h to allow the knockdown of MRGPRX2. These cells were then used for the β -hexosaminidase assay and histamine release assay.

2.14. Western blot analysis

Total protein from untreated and treated LAD2 cells (24 h) was extracted on ice for 30 min using RIPA lysis buffer containing 10% protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics). Insoluble protein lysate was removed by centrifuging the samples at 13,500g for 10 min at 4 °C. The protein concentration was determined using a BCA Protein Quantification kit according to the manufacturer's instructions. The protein in the cell lysates was denatured by boiling the samples with a 5 $\times$ loading sample buffer from Thermo Fisher Scientific, Inc. (MA, USA) for 5 min, and equal amounts of protein were then separated on a 10% SDS-PAGE gel (Shaanxi Pioneer Biotech Co., Ltd.). Following the electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride membranes from Hangzhou Microna Membrane Technology Co., Ltd. (Hangzhou, China), which were blocked with 5% non-fat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd.) for 2 h at room temperature with continuous agitation. The membranes were then incubated overnight at 4 °C with the following primary antibodies: anti-PLC γ 1 (1:1000, #5690, Cell Signaling Technology [CST]), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (1:1000, #8713, CST), anti-IP3R (1:1000, #3763, CST), anti-phosphorylated-IP3R (P-IP3R, Ser1756) (1:1000, #8548, CST), and anti-GAPDH (1:2000, #2118, CST). The membranes were then washed three times with TBST (10 min per wash) followed by incubation with secondary antibodies at a dilution of 1:20,000 in TBST for 1 h at 37 °C, after which the membranes were washed three times with TBST (10 min per wash) and developed using an enhanced chemiluminescence (ECL) kit. A Lane 1DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to image the developed blots, and the Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

2.15. Statistical analysis

Experiments were repeated 3 times. Data are expressed as mean $\pm$ S.E.M. and analyzed using two-tailed unpaired student's *t*-test. An independent samples analysis of variance was used to determine statistical significance in comparisons of the data using the SPSS software. Differences were considered significant at **p* < 0.05, ***p* < 0.01, ****p* < 0.005.

3. Results

3.1. Mivacurium induces mast cell degranulation in a dose-dependent manner

Mivacurium caused human mast cell (LAD2) degranulation *in vitro*,

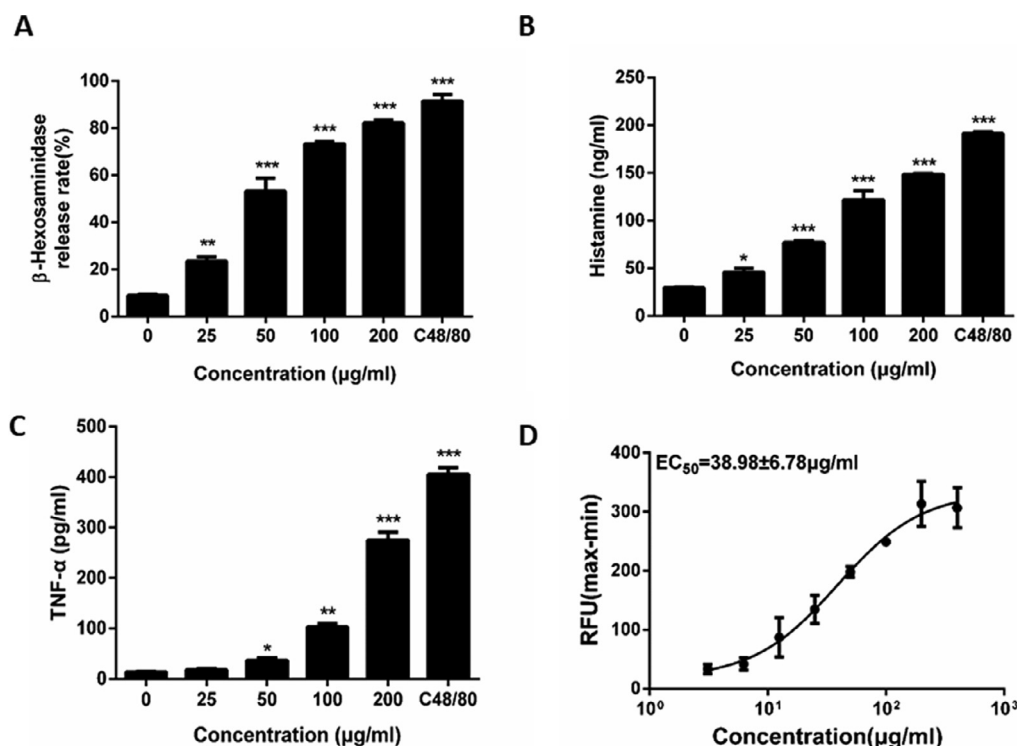


Fig. 1. Mivacurium induced LAD2 mast cell degranulation. (The dose of mivacurium was set as 25, 50, 100, 200 μg/ml and TM buffer was set as negative control and 30 μg/ml C48/80 as positive control.) (A) β-Hexosaminidase release from LAD2 cells treated with different concentrations of mivacurium. (B) Histamine release from LAD2 cells treated with different concentrations of mivacurium. (C) TNF-α release on LAD2 cells treated with serial concentration of mivacurium. (D) The EC_{50} value of mivacurium. Experiments were repeated 3 times, and data are expressed as mean $\pm$ S.E.M. and analyzed using two-tailed unpaired student's *t*-test. Differences were considered significant at **p* < 0.05, ***p* < 0.01, and ****p* < 0.005.

which led to the dose-dependent release of β-hexosaminidase and histamine (Fig. 1A and B). Doses of 50, 100, and 200 μg/ml mivacurium were used; TM buffer was used as a negative control, and compound 48/80 was used as a positive control. The release rate of β-hexosaminidase for the three mivacurium doses was $56.45\% \pm 2.43$, $72.74\% \pm 3.73$, and $80.15\% \pm 2.26$, respectively. The amount of histamine released was 81.32 ± 4.98 , 128.63 ± 7.43 , and 147.86 ± 6.65 ng/ml, respectively, and the amount of TNF-α released was 34.33 ± 2.53 , 101.75 ± 8.84 , and 287.32 ± 11.28 pg/ml, respectively, and the effect exhibited dose dependency (Fig. 1C). HEK293 cells that overexpressed MRGPRX2 were used to determine the half-maximal effective concentration (EC_{50}) of the drug tested. The EC_{50} value of mivacurium was 39.89 ± 6.78 μg/ml (Fig. 1D). Mivacurium activated HEK293 cells overexpressing MRGPRX2 and increased the intracellular Ca^{2+} concentration. The ability of mivacurium to activate the MRGPRX2-overexpressing HEK293 cells was strong.

3.2. Mivacurium induced pseudo-allergic reactions in mice via *Mrgprb2*

For the STOCK Kit^{W-sh}/HNIhrJaeBsmJNju mice in this study, the paw swelling and EBD exudation were similar to those observed in the negative control (*p* > 0.05) (Fig. 2A and B). Mivacurium could not induce a pseudo-allergic reaction in mast cell-deficient mice. Therefore, we hypothesized that mivacurium leads to pseudo-allergic reactions via mast cells.

Further *in vivo* analysis showed that the degree of swelling and EBD exudation in the left limbs of the C57 wild-type mice injected with the drug were higher than those observed in the right limbs, which were injected with a physiological saline solution, and that these effects were directly proportional to the drug concentration (Fig. 2C and D). Additionally, the 0.75-mg/ml dose of mivacurium induced a degree of

swelling and EBD exudation that was higher than that induced by compound 48/80. These experiments revealed that mivacurium could induce local pseudo-allergic reactions in wild-type mice. Since the human MRGPRX2 gene is homologous to the mouse *Mrgprb2* gene, a comparison between wild-type C57 mice and *Mrgprb2*-knockout mice was performed to further elucidate the target involved in the drug-induced pseudo-allergic reactions. The paws of the knockout mice injected with this drug did not swell and showed no obvious EBD exudation or local pseudo-allergic reactions (Fig. 3A and B). Mivacurium was then administered to the mice systemically via tail vein injection, and body temperature changes in the mice were monitored (Fig. 3C). The results showed that mivacurium significantly reduced the body temperature of the mice, however, the reduction in body temperature was insignificant, with no allergic reaction in the knockout mice compared to the responses observed in the wild-type mice. Primary peritoneal mast cells from the wild-type and *Mrgprb2*-knockout mice were also treated with the drug. The results showed that mivacurium elevated the intracellular Ca^{2+} concentrations in more than 80% of the primary peritoneal mast cells from the wild-type mice, whereas only approximately 10% of the primary peritoneal mast cells from the *Mrgprb2*-knockout mice showed elevated Ca^{2+} levels. The difference between the Ca^{2+} result from the wild-type mice and that from the *Mrgprb2*-knockout mice was significant (Fig. 3D). Furthermore, the results of the skin pseudo-allergic reaction and HE staining indicated that mivacurium could induce obvious angiectasis in the skin of the wild-type C57 mice. In contrast, the drug had less of an impact on the *Mrgprb2*-knockout mice (Fig. 4).

The results revealed that mivacurium interacts with MRGPRX2 and triggers a mast cell response, leading to pseudo-allergic reactions.

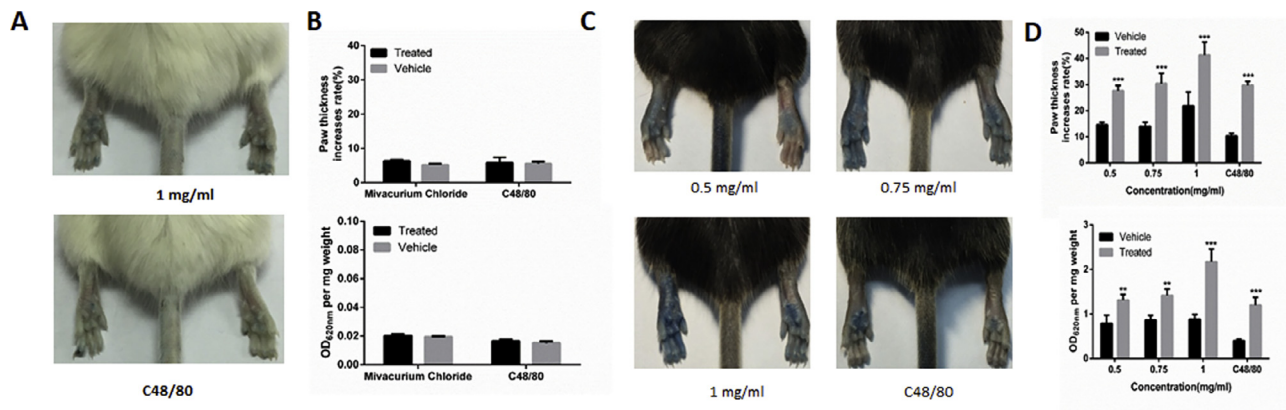


Fig. 2. Mivacurium increased anaphylactoid reactions in wild type C57 BL/6 mice ($n = 6$ per group; experiments were repeated > 3 times. The data are presented as the means $\pm$ s.e.m. Two-tailed unpaired Student's t -test). Anaphylactoid reactions of mice that were treated with 1 mg/ml mivacurium, and 30 μ g/ml C48/80 as positive control. Left, representative image of Evans blue dye (EBD) extravasation 15 min after intraplantar injection of different concentrations of substances (left) or saline (right). Right, the quantification of EBD leakage into the paw and the paw thickness increase after 15 min. (A-B) Mivacurium did not increase anaphylactoid reactions in the $\text{Kit}^{\text{W-sh/W-sh}}$ mice. (C-D) Mivacurium induced pseudoallergic reactions in the C57 wild-type mice.

3.3. Mivacurium induced the release of pre-synthesis inflammatory molecules

Chemokine release can be very harmful to patients and may induce delayed allergic reactions. We analyzed the chemokine release via ELISA spot analysis, and the results showed that mivacurium induced degranulation *in vitro*. However, the induced chemokine release was similar to that in the negative control ($p > 0.05$) (Supplementary Fig. 1), except for $\text{TNF-}\alpha$ (Fig. 1C). The amount of $\text{TNF-}\alpha$ released was 34.33 ± 2.53 , 101.75 ± 8.84 , and 287.32 ± 11.28 pg/ml for the mivacurium doses of 50, 100, and 200 μ g/ml, respectively, which revealed a dose-dependent effect. The release of many chemokines, such as IL-8, MCP-1 and MIP-1d, was not induced by mivacurium. It may be

that mivacurium induces the release of pre-synthesis inflammatory molecules, such as histamine and $\text{TNF-}\alpha$, during the perioperative period and would not result in subsequent harm to a patient.

3.4. MRGPRX2 mediated the mivacurium-induced mast cell activation

Further experimental study revealed that 100 μ g/ml mivacurium increased the intracellular Ca^{2+} concentration in HEK293 cells that overexpressed MRGPRX2 but did not alter the intracellular Ca^{2+} concentration in HEK293 cells transfected with an empty plasmid (Fig. 5A).

Transfection with siRNA was used to verify the target of mivacurium. MRGPRX2 in LAD2 human mast cells was down-regulated using siRNA technology, while LAD2 cells transfected with

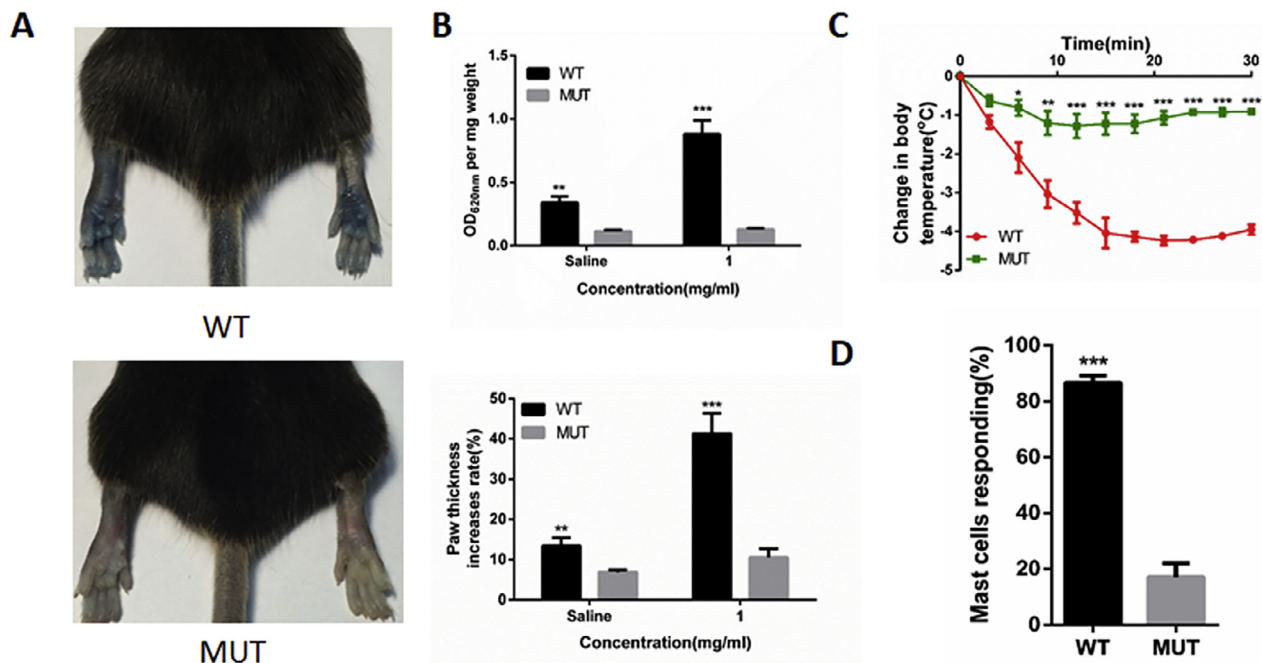


Fig. 3. Mrgrpr2-mediated mast cell responsiveness and anaphylactoid reaction to mivacurium ($n = 6$ per genotype; experiments were repeated > 3 times. The data are presented as the means $\pm$ s.e.m. Two-tailed unpaired Student's t -test). Mrgrpr2 $^{-/-}$ (MUT) mice were injected into 1 mg/ml of mivacurium in the left paw and saline was administered in the right paw as a negative control, 30 μ g/ml C48/80 as positive control (A) Representative images of EBD extravasation 15 min after intraplantar injection of mivacurium. (B) The histograms showed quantification of Evans blue leakage into the paw and paw thickness increase. (C) Change in body temperature of wild-type (WT) and Mrgrpr2 $^{-/-}$ (MUT) mice after the tail vein injection of 0.25 mg/kg mivacurium. (D) Percentage of responding cells from wild-type (WT) and Mrgrpr2 $^{-/-}$ (MUT) mice peritoneal mast cells after drug application, assayed using Fluo-3 imaging.

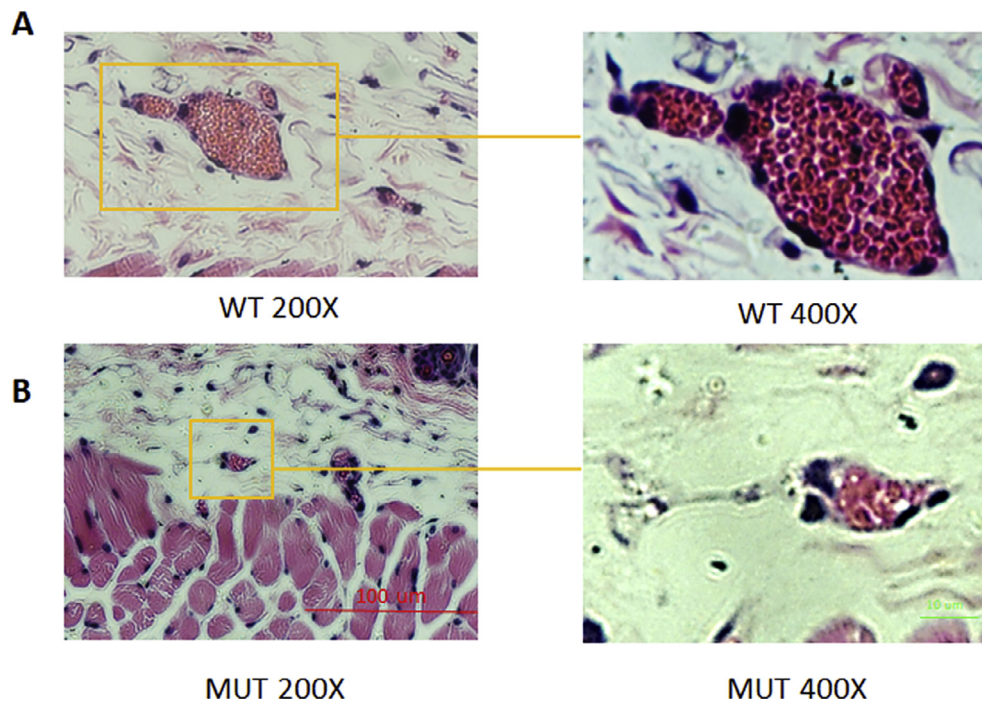


Fig. 4. HE staining of the skin of wild-type (WT) and *Mrgprb2*^{-/-} (MUT) mice. (A) The subcutaneous injection of mivacurium in WT mice induced hemangiectasis. (B) The subcutaneous injection of mivacurium in MUT mice did not induce hemangiectasis (n = 6 per genotype; experiments were repeated 3 times.).

nonfunctional siRNA were used as a negative control (Supplementary Fig. 2). The rate of β -hexosaminidase release and the amount of histamine released by LAD2 mast cells were determined 30 min after the administration of the drug (Fig. 5B and C). At the same drug concentration, the amounts of β -hexosaminidase and histamine released by the MRGPRX2-knockdown LAD2 cells were lower than those of the NC-LAD2 cells, with a significant decrease in drug-induced degranulation. A further experiment confirmed that the mivacurium-induced release of

pre-synthesis TNF- α from LAD2 cells was significantly higher than that from MRGPRX2-knockdown LAD2 cells (Fig. 5D). These results showed that mivacurium induces degranulation via MRGPRX2.

3.5. MRGPRX2 mediates mivacurium-triggered calcium mobilization during anaphylactic reactions by activating PLC- γ and IP3R

After treatment with 50 μ g/ml, 100 μ g/ml and 200 μ g/ml

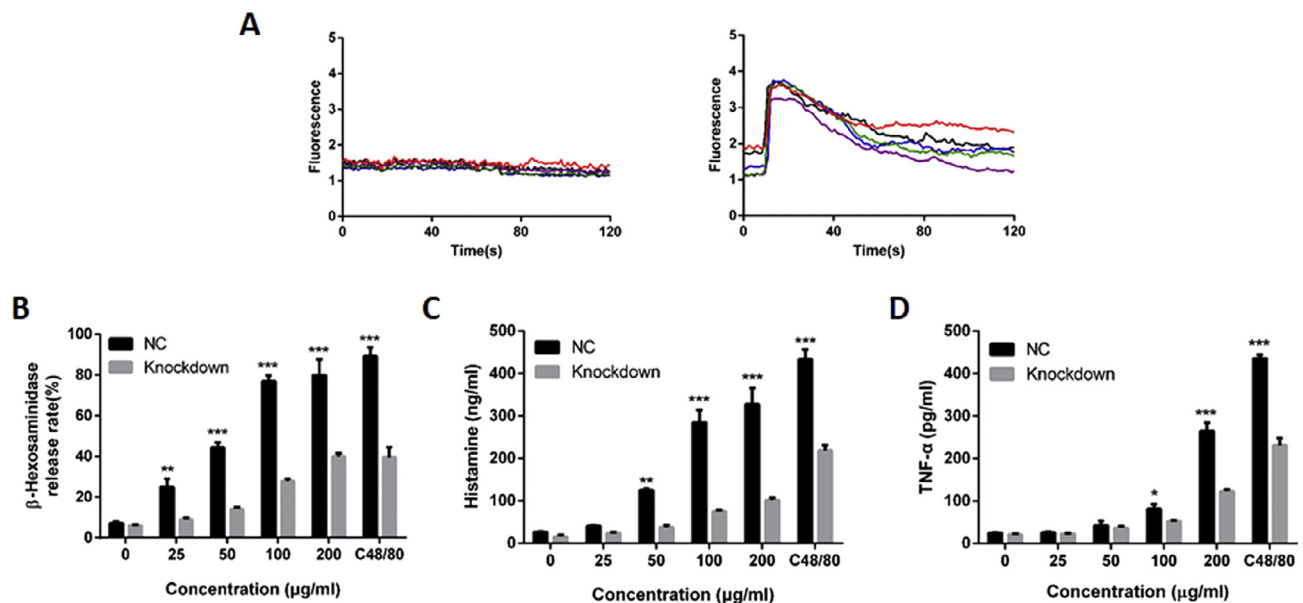


Fig. 5. Mivacurium induced mast cell degranulation via MRGPRX2. (A) Mivacurium did not increase intracellular calcium ion (Ca^{2+}) concentrations in NC-HEK293 cells, while intracellular Ca^{2+} concentrations were increased in cells overexpressing MRGPRX2. Example traces of intracellular calcium concentrations [Ca^{2+}]_i, measured by Fluo-3 imaging, from MRGPRX2-HEK and NC-HEK cells. Each colour trace is a response from a unique cell. (B-C) β -hexosaminidase and histamine release in NC-LAD2 and MRGPRX2-knockdown LAD2 cells treated with mivacurium (The dose of mivacurium was set as 25, 50, 100, 200 μ g/ml and TM buffer was set as negative control and 30 μ g/ml C48/80 as positive control). (D) NC-LAD2 and *MrgprX2*-knockdown LAD2 cells treated with mivacurium. Experiments were repeated 3 times, and data are expressed as mean $\pm$ S.E.M. and analyzed using two-tailed unpaired student's *t*-test. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$.

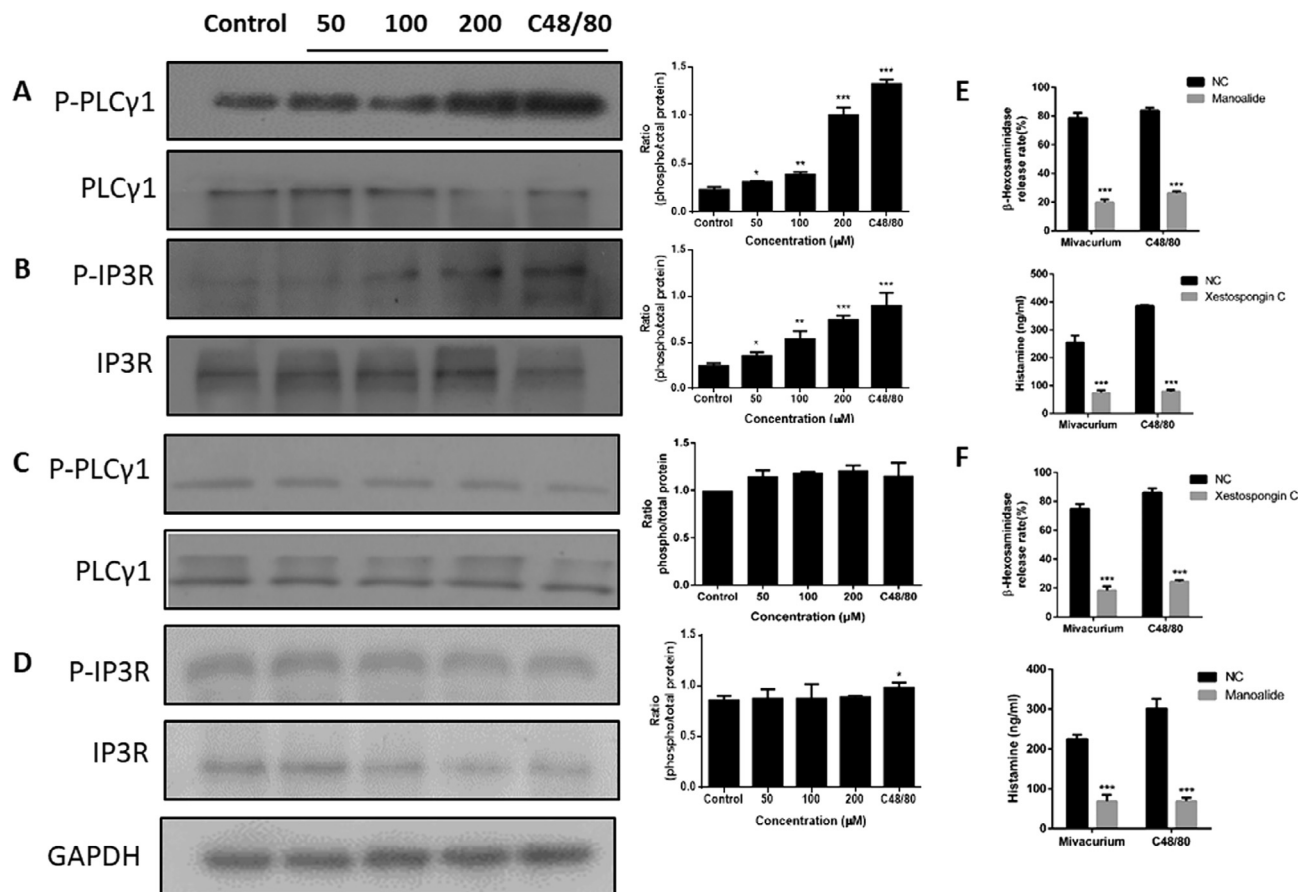


Fig. 6. The molecular mechanism of mivacurium-induced anaphylactic reactions via MRGPRX2 (the dose of mivacurium was set as 50, 100, 200 μg/ml and TM buffer was set as negative control and 30 μg/ml C48/80 as positive control). (A-B) MRGPRX2 mediates mivacurium-triggered calcium mobilization during anaphylactic reactions by activating PLC-γ and the IP3R. (C-D) Mivacurium had no effect on PLC-γ and IP3R after the knockdown of the MRGPRX2 gene in LAD2 cells. (E-F) The release of β-hexosaminidase and histamine in treated LAD2 cells caused by 200 μg/ml mivacurium reduced compared with the negative control cells after blocking PLC-γ and IP3R using Manoalide and Xestospong C, respectively (Experiments were repeated 3 times, and data are expressed as mean ± S.E.M. and analyzed using two-tailed unpaired student's *t*-test. Differences were considered significant at **p* < 0.05, ***p* < 0.01, and ****p* < 0.005).

mivacurium or compound 48/80 for 24 h, the phosphorylation level of key proteins in calcium pathways downstream of MRGPRX2 in LAD2 cells was increased with a dose-dependent manner (Fig. 6), indicating that mivacurium activated MRGPRX2 in LAD2 cells. PLC-γ hydrolyzes phosphatidylinositol-4,5-bisphosphate to soluble inositol-1,4,5-trisphosphate (IP3). The binding of IP3 to the IP3R causes the “first wave” of calcium mobilization, which is the transient release of Ca²⁺ from endoplasmic reticulum stores. The results showed that the treatment of LAD2 mast cells with 50 μg/ml, 100 μg/ml and 200 μg/ml mivacurium for 24 h increased the levels of phosphorylated PLC-γ (P-PLC-γ) and phosphorylated IP3R in a dose-dependent manner (Fig. 6A and B). Therefore, we investigated whether mivacurium activates PLC-γ in siRNA-transfected LAD2 cells using western blot analysis. MRGPRX2-knockdown LAD2 cells were treated with 50 μg/ml, 100 μg/ml or 200 μg/ml mivacurium for 24 h, after which the levels of P-PLC-γ were determined. The results showed that when the MRGPRX2 gene was knocked down, mivacurium had no effect on PLC-γ, P-PLC-γ, IP3 or P-IP3 protein expression (Fig. 6C-D). Further experiments showed that after blocking PLC-γ and IP3R using Manoalide and Xestospong C (Abcam Trading Company Ltd., Shanghai, China), respectively, the release of β-hexosaminidase and histamine in LAD2 cells caused by mivacurium reduced compared with the negative control (Fig. 6E-F).

4. Discussion

Allergic reactions during anesthesia in the perioperative period

present a widespread risk for patients, and the most commonly involved agents are NMBA (61.8%), antibiotics (14.5%), latex (9.2%) and chlorhexidine (5.2%) [22]. NMBAs are responsible for more than two-thirds of the anaphylactic or anaphylactoid reactions and 80% of the cases of anaphylactic shock during anesthesia [23]. Mivacurium is widely used in many types of surgeries, particularly in pediatric anesthesia, because this drug is the shortest acting nondepolarizing muscle relaxant currently available. Several diagnostic techniques for detecting NMBA-induced anaphylaxis have been developed based on IgE-mediated allergic reactions. However, mivacurium is able to induce anaphylaxis via an IgE-independent pathway. Recently, the G-protein-coupled receptor MRGPRX2 has been identified as the receptor responsible for NMBA-induced anaphylactoid reactions. Our study revealed that mivacurium induced mast cell degranulation in a dose-dependent manner (Fig. 1A and B). Furthermore, mivacurium was able to induce local pseudo-allergic and systemic pseudo-allergic in mice, including swelling caused by histamine release and a decrease in body temperature (Fig. 2B, Fig. 3B and Fig. 4). Further studies employing siRNA transfection and Mrgprb2-knockout mice found that mivacurium induced pseudo-allergic by activating mast cells via the MRGPRX2 receptor (Fig. 2A, Fig. 3A, B and Fig. 5). Additionally, the results of western blot analyses showed that MRGPRX2 mediates mivacurium-triggered calcium mobilization during pseudo-allergic reactions via the activation of PLC-γ and IP3R (Fig. 6).

There have actually been few reports regarding serious anaphylactic reactions induced by mivacurium in the clinic. However, the EC₅₀ value

of mivacurium indicated that this drug can strongly activate MRGPRX2 (Fig. 1C), which contradicts the lack of reports. Our current study, however, has provided an explanation for this phenomenon. The mivacurium-induced pseudo-allergic reactions that occur via MRGPRX2 only result in the release of pre-synthesis inflammatory molecules, such as histamine and TNF- α , it does not induce the release of more dangerous chemokines, such as interleukin, which might cause severe harm to the patient. TNF- α and histamine will be dangerous to patients but mivacurium does not appear to induce other chemokine release, which might cause severe harm to the patient. Pre-synthesis inflammatory mediators, such as histamine, only appeared after mivacurium was administered in the perioperative period. Based on the findings of the present study, anesthesiologists should be more aware of the anaphylactoid reactions caused by histamine and only use mivacurium in patients undergoing surgery who do not pose a risk of allergy in the postoperative recovery period.

Mivacurium is a steroidal non-depolarizing aminosteroid neuromuscular blocking agent that couples with the acetylcholine receptor to reduce or inhibit the depolarizing effect of acetylcholine on the terminal disc of the muscle cell [24] and can be used in short-duration surgical procedures. When mivacurium and rocuronium bromide are both suitable for a surgery, the safety of the drug will be a key factor for choosing an NMBA. The basophil activation test and the skin test have both been used to evaluate the sensitivity to rocuronium bromide, and the results showed that rocuronium bromide can induce severe anaphylactic reactions [25]. Rocuronium bromide has caused severe anaphylactic reactions in the clinic and has even resulted in patient death [26,27]. Rocuronium bromide-induced anaphylaxis may develop through an IgE-mediated allergic reaction. Recent studies, as well as our study, suggest that mivacurium might be a safer alternative to rocuronium bromide.

In clinical, mivacurium injection can be used at the concentration of 2 mg/ml which is higher than the concentration induced pseudo-allergic reactions. But few case about mivacurium induced pseudo-allergic reactions were reported. Mivacurium is highly correctly with concentration in serum. And Hofmann rearrangement that may be the reason why the pseudo-allergic reaction in rare case cause in clinical. Even though the concentration of mivacurium in serum in clinical use is lower than the dose in our research, mivacurium also induces pseudo-allergic reactions. Our research suggested the dosage regimen and dose rate are the key factors to reduce the pseudo-allergic reactions induced by mivacurium. The results of this study suggest that if anesthesiologists who use mivacurium paid more attention to the possible pseudo-allergic reactions caused by histamine, then patients undergoing surgery would not risk allergic reactions during the postoperative recovery period. The results of this study might offer a novel approach to the design of new types of muscle relaxants that avoid an allergenic effect. The targeting of human MRGPRX2 might also offer a promising way to avoid drug-induced anaphylactoid reactions to the muscle relaxant mivacurium.

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Disclosures

The authors have no financial conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.cellimm.2018.08.005>.

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