SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Assessment of function of the neuromuscular junction in phrenic nerve-diaphragm preparation

by Dr. Vera Csernoch

Supervisors: Professor Dr. Béla Fülesdi, DSc

Dr. Ákos István Fábián, PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF NEUROSCIENCE

DEBRECEN, 2024

Assessment of function of the neuromuscular junction in phrenic nerve-diaphragm preparation

By Dr Vera Csernoch, Anesthesiologist and Intensive Therapy specialist (MD/MSc degree)

Supervisors: Professor Dr. Béla Fülesdi, PhD, DSc

Dr Ákos István Fábián, PhD

Doctoral School of Neuroscience, University of Debrecen

Head of the **Examination Committee**: Professor Dr. Endre Nagy, PhD, DSc

Members of the Examination Committee:

Dr. Álmos Klekner, PhD, DSc

Professor Dr. Katalin Darvas, PhD

The Examination takes place at Departement of Internal Medicine Building A, Library, Faculty of Medicine, University of Debrecen, 2024. May 28. 11 am.

Head of the **Defense Committee**:

Professor Dr. Endre Nagy, PhD, DSc

Reviewers:

Professor Dr. István Bátai, PhD

Dr. Klára Edit Fekete, PhD

Members of the Defense Committee:

Dr. Álmos Klekner, PhD, DSc

Professor Dr. Katalin Darvas, PhD

The PhD Defense takes place at the Lecture Hall of Departement of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen, 2024. May 28., 1 pm

Table of Contents

1. Introduction

Since the introduction of the curare in 1942, neuromuscular blocking agents have allowed immobilisation of patients during surgery, improved the feasibility of surgical procedures and improved tracheal intubation conditions. However, the use of neuromuscular blocking agents has increased the risk of mortality by a factor of six, demonstrating the increased risk associated with the use of muscle relaxants. Many improvements have been made over the decades to increase patient safety when using muscle relaxants under general anaesthesia. Several new neuromuscular blocking agents have been synthesised and developed to create the most ideal muscle relaxant effect. Instruments for qualitative and quantitative monitoring of muscle function have been developed. To revers the residual muscle relaxant effect, the use of neostigmine was introduced in the 1950s and for decades it was the only reversal agent available to clinicians. The numerous side-effects of the acetylcholinesterase inhibitor and its inability to revers deep muscle blocks make it far from the ideal of the perfect reversal agent. In 2008, a new type of selective relaxant binding agent, sugammadex, a γ-cyclodextrin derivative, appeared in the clinical use. This compound has the ability to rapidly and completely revers muscle blocks of any depth, but it has its limitations. It is only effective on aminosteroid muscle relaxants and among other things, anaphylactic reactions and bradycardia has been reported with its use. The invention of the ideal muscle relaxant reversal agent is therefore still to be found. Many innovations over the last 70 years have reduced the direct mortality from neuromuscular blocking agents, but the risk of respiratory complications remains. To this day, the incidence of postoperative residual neuromuscular block is estimated to be between 2–64%. Undesirable effects of residual muscle relaxation include hypoxia, respiratory depression, bronchoaspiration, atelectasis, pneumonia, or even death. It is therefore of great

importance that anaesthetists ensure that full muscle strength is restored at the end of surgery and that no residual muscle relaxant effect remains, which may increase patient mortality and morbidity.

To study the effect, in addition to clinical trials, the n. phrenicus diaphragm preparation can be used in the preclinical phase. The isolated rat phrenic nerve-diaphragm preparation has proved to be an excellent method for the realisation of numerous muscle physiology and pharmacology experiments, even today. To the best of our knowledge, such a preparation can be tested in Europe at the Neuromuscular Research Group of the Technische Universität in Munich and at the Neuromuscular Working Group of our clinic. In the experimental laboratory of our clinic, we have used this preparation for studies that can be used later in the clinical practice.

2. Aimes

In ex vivo animal experiments using an isolated rat phrenic nerve-diaphragm tissue preparation, we investigated the effects of muscle relaxants and their reversibility in two different series of experiments.

The aim of our first series of experiments was to investigate the efficacy of a new γ-cyclodextrin derivative, carboxymethyl-γ-cyclodextrin, on the reversal of three muscle relaxants in clinical use, rocuronium, vecuronium and pipecuronium. Our further objective was to compare the efficacy of carboxymethyl-γ-cyclodextrin with that of another γ-cyclodextrin derivative, sugammadex, already on the market and in clinical practice.

In our second series of experiments, also performed on the ex vivo rat phrenic nerve-diaphragm preparation, we investigated the effects of different magnesium concentrations on rocuronium-induced muscle relaxation and its reversibility by sugammadex. Our aim was to determine the effect of increased magnesium concentration on rocuronium-induced muscle relaxation, on its reversibility and on recurarisation.

3. Methodologies

3.1. Experimental animals

Both sets of experiments were performed on rat preparations. Ethical approval for the research (1/2013/DE MÁB) was granted by the University of Debrecen Animal Experiments Scientific Ethics Board (President Prof Furka I.) on 15 April 2013. For the two studies 20–20 male Wistar rats weighing 250–563 g per study were used. Institutional guidelines on the proper treatment of animals and their use for research purposes were strictly adhered to. The animals were randomly selected on the morning of the experiments and euthanized immediately before the experiments started.

3.2. Materials

3.2.1. Study of the effect of carboxymethyl-γ-cyclodextrin

In these experiments, rocuronium (Esmeron; MSD Pharma Hungary, Budapest, Hungary), pipecuronium (Arduan; Richter Gedeon, Budapest, Hungary), vecuronium (Vecuronium Inresa; Inresa Arzneimittel Ltd, Freiburg, Germany), and sugammadex (Bridion; MSD Pharma Hungary, Budapest, Hungary) were used, purchased commercially and dissolved in Krebs solution in a volume sufficient to allow dosing in 10-100 μL volumes.

In addition, a new γ-cyclodextrin derivative, carboxymethyl-γ-cyclodextrin (CMGCD), developed and manufactured by Cyclolab Ltd, Budapest, Hungary, was used for the experiment. For

the synthesis of the compound, γ-cyclodextrin was dissolved in an aqueous solution of sodium chloroacetate. The resulting solution was heated to 70°C. When the temperature reached 60°C, aqueous NaOH was added dropwise to the solution over 3 h, and the solution was further stirred at 75°C for a further 4 h. Subsequently, when the reaction mixture had cooled back to room temperature, it was treated with a strong ion exchange resin and the filtrate was carbon purified. The colourless solution was finally lyophilised. The resulting CMGCD variants differ in the degree of substitution (DS), the extent of which was determined by nuclear magnetic resonance spectroscopy (NMR) and capillary electrophoresis (CE). Based on preliminary ex vivo data, we used CMGCD DS = 4.1 variant for our experiments.

3.2.2. Examination of the effect of magnesium

In this series of experiments, we used rocuronium (Esmeron; MSD Pharma Hungary, Budapest, Hungary) and sugammadex (Bridion; MSD Pharma Hungary, Budapest, Hungary), which were commercially purchased and dissolved in Krebs solution in a volume sufficient to allow dosing in 10–100 μL volumes.

In addition, magnesium heptahydrate sulphate (Cormagnesin, Wörwag Pharma GmbH, Böblingen, Germany) was used and added to the buffer solution without dilution to achieve the appropriate final concentration of magnesium.

3.3. Experimental methods

3.3.1. Implementation of the ex vivo rat tissue preparation

Rat phrenic nerve-hemidiaphragm preparations were used for both sets of experiments. The rats were killed with overdosed

thiopental (60 mg/kg) injected into the peritoneum and exsanguinated through on incision on the dorsal vena cava. For hemidiaphragm preparation, a modified version of the method described by Bülbring was used. A bilateral thoracotomy was performed and the sternum was then excised. The two phrenic nerves were then dissected from the cranial direction in a rostral direction until they were connected to the diaphragm. The two hemidiaphragms were then dissected out with their respective phrenic nerves. After preparation, the nervus phrenicus-hemidiaphragm preparations were placed in a special tissue holder (IS0-07-TSZ2D, Experimetria Ltd, Budapest, Hungary), in 75 mL Krebs solution (110 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 , 5 mM glucose, 20 mM $NaHCO_3$), which was bubbled with 95% O_2 and 5% CO_2 (Vol%). The solution was kept at 37 °C. (AMP-09 Temperature controller, Experimetria Ltd., Budapest, Hungary).

The hemidiaphragms were attached to an isometric force transducer (FSG-01/200 Force Transducer, Experimetria Ltd., Budapest, Hungary) at the diaphragm centrum tendon with a 5/0 diameter surgical suture. The measurements were amplified with an AMP-01-SG Classic bridge amplifier and recorded with a 16-channel professional software package (S.P.E.L. Advanced Isosys software, Experimenta Ltd., Budapest, Hungary). The phrenic nerve was stimulated either with single twitch (ST) every 5 s (rectangular supramaximal pulses of 0.3 ms duration) or with a train-of-four (TOF) stimulation pattern every 15 s (four consecutive rectangular supramaximal pulses of 0.2 ms duration, delivered at 2 Hz frequency, repeated every 15 s). Electrical stimulation was delivered using a square pulse generating electrical stimulator (ST-03-O4, Experimenta Ltd, Budapest, Hungary).

After placing the preparation in the buffer solution, the tissues were allowed to acclimatise for 10 min, during which time no nerve stimulation was applied, only a basic 20–30 mN tension was used. Electrical stimulation of the phrenic nerve was then started and continued for 1–1.5 hours, while no other agents

were added to the solution, only the buffer solution was changed as needed, until a stable basal tension was reached. Muscle relaxants, selective relaxant binding agents, and magnesium were added to the solution only after the stabilization period. After a given concentration-response curve was measured, the buffer solution was changed 5 times over a 30 min time interval to ensure complete washout of the substances added to the solution before starting a new concentration-response curve measurement.

Several measurements were made on a given rat, but one animal contributed only one measurement to a concentration-response curve. In order to reduce the effects of degradation over time in the tissue, the concentration-response curves were recorded in the animals in a varied order. Preparations were discontinued when a stable base strain could no longer be maintained. Each concentration-response curve is derived from concentration-response curves recorded from 5 different animals.

3.3.2. Study of the effect of carboxymethyl-γ-cyclodextrin

3.3.2.1. Concentration-response curves for rocuronium, vecuronium and pipecuronium

In this series of experiments we first recorded the concentration-response curves of three muscle relaxants, rocuronium, vecuronium and pipecuronium. The effect of the muscle relaxants was measured by the reduction in the strength of muscle contractions induced by electrical impulses in the form of single twitches (ST) on the phrenic nerve (hereafter referred to as ST force amplitude). The neuromuscular blocking agents were administered at 15 minute intervals. ST force amplitude was calculated from the average of five consecutive muscle contractions at a given drug concentration, when the contraction amplitudes had already stabilized and did not change visually over time. Values

were corrected to the baseline strain measured between contractions and normalized to the maximum contraction amplitude of the untreated preparation to generate cumulative concentration-response curves. Each preparation provided 5–8 measurement points on the curve.

3.3.2.2. Concentration-response curves of sugammadex and CMGCD during aminosteroid muscle relaxant-induced muscle block reversion

In order to determine the effect of selective relaxant binding agents (SRBA), a single dose of muscle relaxant was added to the buffer solution to achieve a 90–95% reduction in ST force amplitude.

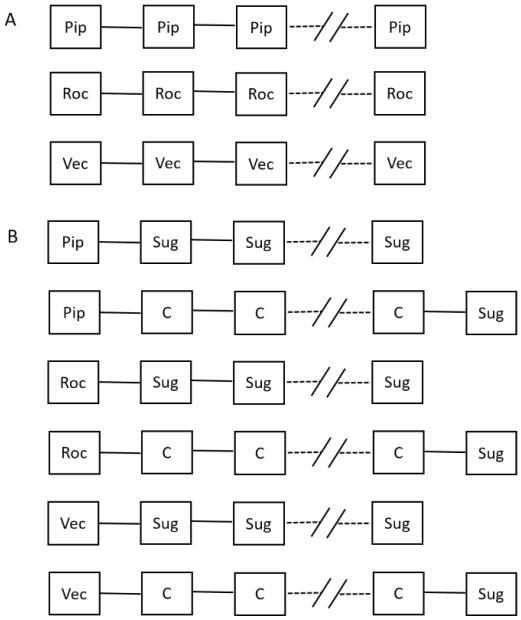

Figure 1. Experimental setup. A: Editing of concentration-response curves for neuromuscular blocking agents. bolus muscle relaxants were administered every 15 min until complete suppression of the ST response was achieved. B: Editing

of concentration-response curves of SRBAs. After administration of an inicial bolus of NMBD to achieve 90–95% ST depression, SRBAs were administered every 15 min until complete reversal of neuromuscular block was achieved. A reversal dose of sugammadex was administered after reversal with C to confirm the achievement of complete reversal. NMBD: neuromuscular blocking agent, ST: single twitch, SRBA: selective relaxant binding agent, Pip: pipecuronium, Roc: rocuronium, Vec: vecuronium, Sug: sugammadex, C: carboxymethyl-γ-cyclodextrin.

Subsequently, doses of SRBA were administered every 15 minutes until further doses no longer caused a further increase in ST force amplitude. A successful reversal was determined by the TOF ratio if the TOF ratio was greater than 90%. The TOF ratio is the ratio (T4/T1) of the fourth (T4) to the first (T1) pulse of the four electrical stimuli. For CMGCD curves, a reversal dose (0.5 mg) of sugammadex was also added to guarantee complete reversal of the respective muscle relaxant. The ST force amplitude was corrected to the ST force amplitude before SRBA administration and normalized to the maximum contraction amplitude after complete reversal to generate cumulative concentration-response curves. Figure 1 shows our experimental design.

3.3.3. Study of the effect of magnesium

3.3.3.1. Magnesium and rocuronium concentration-response curves

We first determined the concentration-response curves of magnesium and rocuronium in our diaphragm-nervus phrenicus preparation. The effects of magnesium and rocuronium on muscle contractile force were quantified by the amplitude depression of the single twitch force. For a given concentration of magnesium and rocuronium, the single twitch force amplitude per measurement was given by the average of five consecutive contractions when the contraction amplitudes had stabilized. The resulting value was then corrected to the base intensity. The single

twitch force amplitude was normalized to the maximum single twitch force amplitude of the untreated preparation to determine the cumulative concentration-response curves. The magnesium concentration-response curve was measured by injecting 9.2 mg of magnesium into the buffer solution every 10 min until complete depression of the ST force amplitude was achieved. Each preparation contributed 11–12 measurement points to the curve. To determine the concentration-response curve for rocuronium, the compound was added to the solution every 15 min. For the muscle relaxant, two curves were determined at two different magnesium concentrations. In one case, the concentration-response curve for rocuronium was plotted at a magnesium concentration of 1 mM, and in the other case at a magnesium concentration of 1.5 mM. For the 1 mM magnesium concentration, the initial dose of rocuronium was 0.2 mg, followed by 0.1 mg doses until contraction was no longer detectable following nerve stimulation. Each preparation contributed 5–7 measurement points to the curve. For the concentration-response curve recorded at a magnesium concentration of 1.5 mM, the first rocuronium dose was 0.1 mg, followed by two 0.05 mg doses of rocuronium, and then continued in 0.025 mg doses until no more muscle contraction could be elicited upon electrical stimulation. Each formulation contributed 7 to 11 measurement points to the curve.

3.3.3.2. Assessing the pre-block effect of magnesium

We performed a series of measurements to determine the effect of different magnesium concentrations applied before the onset of muscle relaxation, i.e. pre-block, on the reversibility of the neuromuscular block. In this series of experiments, we measured the reversibility of rocuronium-induced muscle relaxation with sugammadex at two different magnesium concentrations. At a magnesium concentration of 1.5 mM, a single dose of 0.3 mg of rocuronium was added to the solution, and at a magnesium concentration of 1 mM, a single dose of 0.5 mg of rocuronium was

added to the solution to achieve a single twitch force amplitude depression of 90–95%. Subsequently, sugammadex was added every 10 min to record the concentration-response curve of the compound. The ST force amplitude was corrected to baseline, which was the ST force amplitude prior to sugammadex administration, and normalized to the maximum contraction amplitude after complete reversal to construct cumulative concentration-effect curves. At a magnesium concentration of 1 mM, the initial sugammadex dose was 0.2 mg, followed by four doses of 0.1 mg, then one dose of 0.15 mg, and finally doses of 0.25 mg until the ST force amplitude reached a plateau and the reversal of neuromuscular block was confirmed by a TOFR > 0.9. Each preparation contributed 5–8 measurement points to the concentration-response curve. At a magnesium concentration of 1.5 mM, the starting sugammadex dose was 0.05 mg, followed by three sugammadex doses of 0.1 mg, then one sugammadex dose of 0.15 mg, and finally sugammadex doses of 0.25 mg until ST force amplitude reached a plateau level and the return of full muscle strength was confirmed with TOFR > 0.9. Each preparation contributed 7 measurement points to the concentration-response curve.

3.3.3.3. Assessing the post-block effect of magnesium

In this experiment, we determined the effect of magnesium on muscle relaxation and its reversibility by sugammadex, by increasing the dose after the onset of neuromuscular block reversal, i.e. post-block. In our experiment, 0.5 mg of rocuronium was added to the solution at a magnesium concentration of 1 mM to produce an ST force amplitude reduction of 90–95%. We then started to revers neuromuscular block with doses of 0.1–0.2 mg sugammadex administered every 10 min until the total sugammadex dose reached 0.5 mg. The magnesium concentration in the solution was then increased to 1.5 mM and the sugammadex administration was continued, first with a single dose of 0.15 mg, then with a 0.1 mg dose. Subsequently, we administered the re-

versal agent in 0.25 mg doses until a plateau effect in ST force amplitude was achieved and the return of full muscle strength was confirmed by achieving a TOFR > 0.9. The ST force amplitude was corrected to baseline, which was the ST force amplitude before sugammadex administration, and normalized to the maximum contraction amplitude after complete reversal to construct cumulative concentration-effect curves. Each preparation contributed 6–7 measurement points to the cumulative concentration-response curve.

3.3.3.4. The effect of magnesium on the safety margin of the reversal

In this experiment, we measured how increasing magnesium concentration in solution affects the rocuronium block reversed with sugammadex after the muscle strength has fully recovered from the reversal, i.e. what effect magnesium has on the safety margin of the reversal.

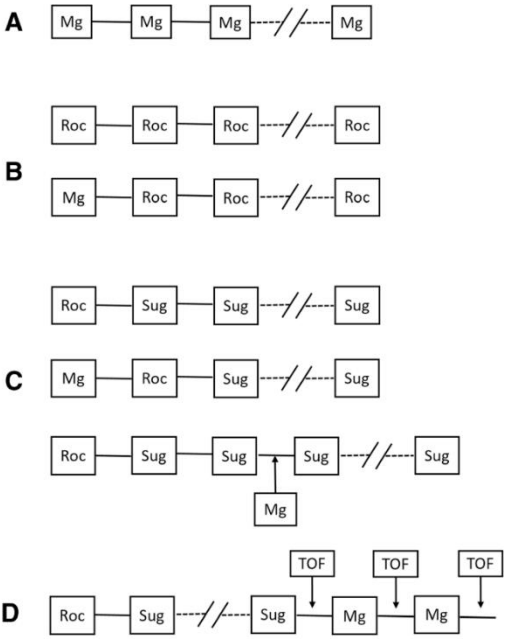

Figure 2. Experiment design. A: Construction of magnesium concentration-response curve. Doses of magnesium were added to the buffer solution every 10 min until complete suppression of ST force amplitude was achieved. B: Construction of rocuronium concentration-response curve. Bolus rocuronium was added every 15 min until complete suppression of ST force amplitude was achieved. To assess the effect of magnesium, the experiment was repeated by adding bolus magnesium to the solution 10 minutes before the start of rocuronium administration. C: Construction of Sugammadex concentration-effect curve. Following administration of an inicial bolus of rocuronium, which caused a minimum 90% ST force amplitude depression, doses of sugammadex were administered every 10 min until complete return of ST amplitude was achieved. In order to assess the effect of magnesium given before inducing muscle relaxation, the experiment was repeated by administering bolus magnesium in solution 10 min before the administration of rocuronium. To determine the effect of magnesium given after the onset of muscle block, the experiment was repeated with bolus magnesium administered after the partial return of muscle strength produced by sugammadex, and sugammadex dosing was then continued. D: Evaluation of the effect of magnesium on the safety margin of the reversal. Following administration of bolus rocuronium, sugammadex doses were administered up to the full, or already considered safe, limit of

muscle relaxation, as measured by TOFR. Magnesium doses were then administered and TOFR was measured after each dose. Mg: magnesium, Roc: rocuronium, Sug: sugammadex, TOF: train-of-four, TOFR: train-of-four rate.

This experiment was performed by reversing the rocuronium-induced muscle block with sugammadex to a TOFR ≈ 1.0 when no visual fatigue was observed, i.e. until full muscle strength was restored, or to a TOFR > 0.9 when visual fatigue was still present but the criterion for clinically safe extubation had been reached. The magnesium concentration was then increased in 0.5 mM increments until a concentration of 2 mM was reached. The TOFR was measured every 15 s for 10 min after each magnesium concentration change, and the final stable TOFR was used to quantify the changes in TOFR. Figure 2 illustrates our experimental design.

3.4. Statistical analysis

In both sets of experiments, we used GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) for MS Windows to fit the concentration-response curves. For curve fitting, the software offers several possible solutions, which are: non-linear regression, "log(agonist) vs. normalized response-variable slope", "log(inhibitor) vs. normalized response-variable slope", "log(agonist) vs. response-Find EC anything". The fitting equation was as follows:

$$y = \frac{100}{1 + (EC50 - X) \cdot Hillslope}, \text{where } X = \log(concetration)$$

and y is the normalized and baseline corrected contraction amplitude. EC_{90} and EC_{99} were determined by the following equation:

$$\log EC50 = \log ECF - \frac{1}{Hillslope} \cdot \log \frac{F}{100 - F}, \text{where } F = 90 \text{ or } F = 99$$

3.4.1. Study of the effect of carboxymethyl-gamma-cyclodextrin-power analysis

Prior to the experiment, a preliminary measurement (n = 3) was made to determine the sample size needed to calculate the pipecuronium concentration-response curve. The results were $logEC_{50}$ = 0.14 and SD = 0.007. Assuming a 10% change in the value of EC_{50}, which can be considered as a clinically relevant change, α = 0.05 and power of 80% sample sizes of n = 5 were obtained to perform a two-sided test. Statistical comparison of the concentration-response curves was performed using the GraphPad Prism 6 program for MS Windows, with an extra sum-of-squares F-test. $LogEC_{50}$, $LogEC_{90}$ or $LogEC_{99}$ was the model component used for the extra sum-of-squares. Results are presented as mean or 95% confidence interval (CI) unless otherwise specified.

Comparisons of muscle relaxant doses before reversal with selective relaxant blocking compound, which produced 90-95% ST force amplitude depression, and the different muscle relaxant concentrations assigned to EC_{50}, EC_{90}, and EC_{99} were compared using Student T-test in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

3.4.2. Examining the effect of magnesium-power analysis

Prior to the experiment, a preliminary measurement (n = 3) was performed to determine the sample size needed to calculate the rocuronium concentration-response curve. In our results we obtained $LogEC_{50}$ = 0.875 and SD = 0.023. Assuming a 10%

change in the value of EC_{50}, which can already be considered a clinically relevant change, $\alpha = 0.05$ and power of 80%, we obtained sample sizes of $n = 4$ to perform a two-sided test. Statistical comparison of concentration-response curves was performed using GraphPad Prism 6 for MS Windows with an extra sum-of-squares F-test. For the extra sum-of-squares we used the model component $LogEC_{50}$. Results are presented as mean or 95% confidence interval (CI).

4. Results

4.1. Study of the effect of carboxymethyl-γ-cyclodextrin

4.1.1. Concentration-effect relationship for different muscle relaxants

As a first step, we determined the concentration-response curves for all muscle relaxants we studied, namely rocuronium, vecuronium, and pipecuronium. In our results, we have indicated the effective concentrations of 50% (EC_{50}) and 90% (EC_{90}), which are the concentrations at which the muscle relaxant effect is normally 50% and 90%, respectively. In the case of rocuronium, EC_{50} was found to be 7.50 μM (6.93–8.12 μM), EC_{90} 11.36 μM (9.64–13.39 μM). For vecuronium EC_{50} was 3.69 μM (3.59–3.80 μM) and EC_{90} 5.29 μM (4.97–5.63 μM). For pipecuronium, EC_{50} was 1.38μM (1.33–1.42 μM) and EC_{90} was 1.68 μM (1.58–1.79 μM). The concentration-response curves for the muscle relaxants are shown in part A of Figure 3 The results showed that pipecuronium showed the highest potency, while vecuronium had a medium potency. Rocuronium required the highest concentration to effectively reduce ST force amplitude in our ex vivo system. Values are presented as mean and in 95% confidence intervals.

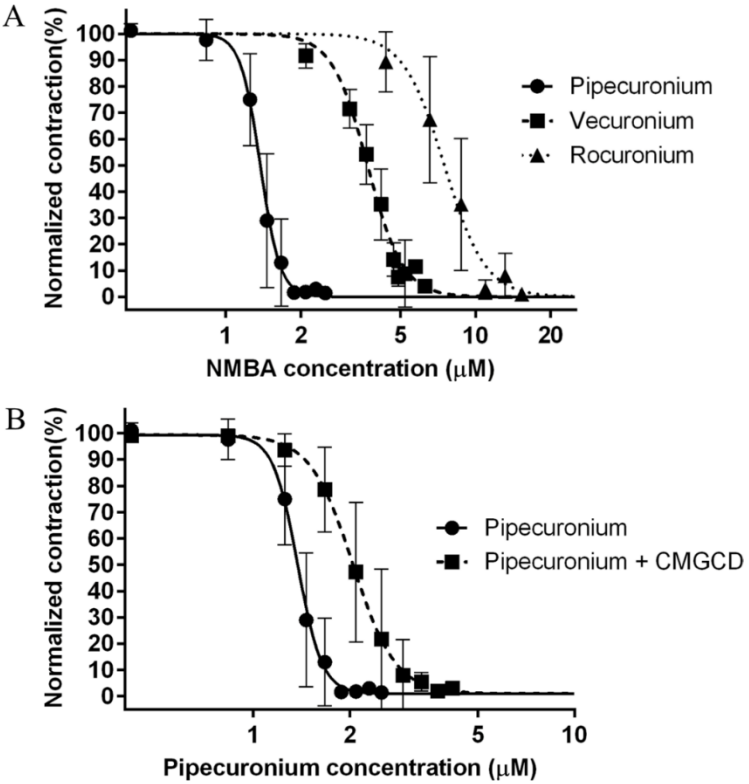

Figure 3. Concentration-response curves for different muscle relaxants. A: Concentration-response curves for pipecuronium, vecuronium and rocuronium. Normalized contraction force amplitude plotted as a function of the logarithm of the decimal of the concentration of the muscle relaxants (log10). Curves are best-fit curves, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points. B: Shows the shift of the pipecuronium concentration-response curve after pretreatment with 13.3 μM CMGCD. CMGCD: carboxymethyl-γ-cyclodextrin, NMBD: neuromuscular blocking drug.

4.1.2. Mechanism of muscle relaxant-induced neuromuscular block reversal by CMGCD

In order to determine the nature of the interaction between CMGCD and muscle relaxants, CMGCD was added to the solution so that its final concentration in the solution was 13.3 μM before increasing the concentration of pipecuronium. After pretreatment with the reversal agent, the EC_{50} of pipecuronium was 2.04 μM (1.94–2.16 μM) and the EC_{90} was 2.90 μM (2.58–3.25 μM). Pretreatment with CMGCD resulted in a rightward shift in the pipecuronium concentration-response curve. However, maximum ST depression was still feasible in the presence of CMGCD, only at higher pipecuronium concentrations (compared to when CMGCD was not present in solution). The concentration-effect curves of the results obtained are illustrated in part B of Figure 3.

Our results suggest that CMGCD binds pipecuronium until its receptors are saturated, after which unbound muscle relaxant molecules are able to induce ST depression.

4.1.3. Potential of CMGCD to revers neuromuscular block compared to sugammadex

CMGCD and sugammadex concentration-response curves were plotted for all three muscle relaxants. For rocuronium, we found that the EC_{50} of CMGCD was 35.89 μM (32.67–39.41 μM), the EC_{90} was 119.9 μM (101.5–141.9 μM), the EC_{50} of sugammadex was 3.67 μM (3.43–3.92 μM) and the EC_{90} was 6.08 μM (5.34–6.92 μM). CMGCD EC_{50} used for the reversal of pipecuronium-induced muscle relaxation was 10.14 μM (9.61–10.70 μM), EC_{90} was 21.60 μM (19.63–23.76 μM), sugammadex EC_{50} was 0.67 μM (0.62–0.74 μM), EC_{90} was 1.05 μM (0.86–1.29 μM). For the vecuronium reversal, we obtained that CMGCD EC_{50} was

376.1 μM (341.9–413.8 μM), EC_{90} was 863.1 μM (727.8–1023 μM), sugammadex EC_{50} was 1.45 μM (1.35–1.56 μM), and EC_{90} was 3.33 μM (2.82–3.92 μM). The concentration-response curves are shown in Figure 4.

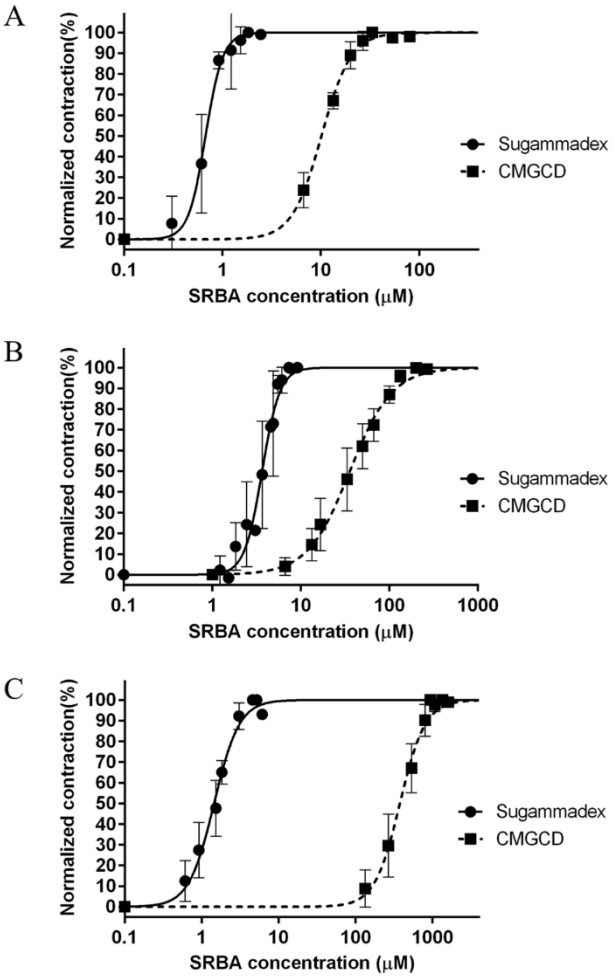

Figure 4. Concentration-response curves for SRBAs. A: Concentration-response curves of sugammadex and CMGCD used to revers Pipecuronium-

induced muscle relaxation. B: Sugammadex and CMGCD concentration-response curves for the reversal of rocuronium-induced muscle relaxation. C: Sugammadex and CMGCD concentration-response curves for the reversal of vecuronium-induced muscle relaxation. Normalized contraction force amplitude plotted as a function of the decimal logarithm of the concentration of SRBAs (log10). Curves are best-fit curves, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points. CMGCD: carboxymethyl-γ-cyclodextrin, SRBA: selective relaxant binding agent.

The EC_{50} and EC_{90} values for both sugammadex and CMGCD were lowest for pipecuronium. The potential of sugammadex to revers the neuromuscular block produced by vecuronium was greater than that produced by rocuronium, whereas CMGCD had a greater potential for rocuronium and had the weakest effect on vecuronium reversal. For all three muscle relaxants, sugammadex had significantly lower EC_{50} and EC_{90} compared to CMGCD (< 0.0001).

While CMGCD was able to achieve complete reversal in pipecuronium-induced muscle relaxation, only 94.6 ± 1.5% muscle force return was achieved with rocuronium and 86.3 ± 5% ST amplitude with vecuronium. Interestingly, for rocuronium-induced block, the ST force amplitude return was greater than the TOF ratio return (94.6% versus 90.6%), whereas for vecuronium, the ST force amplitude return was lower than the TOF ratio return (86.3% versus 87.6%). Although the difference between ST return and TOF rate was not statistically significant ($p = 0.249$ for rocuronium and $p = 0.637$ for vecuronium).

4.1.4. Affinity of selective relaxant binding agents for neuromuscular blocking agents

Because muscle relaxants were added to the solution at concentrations to cause at least 90% ST force amplitude reduction, differences in the potency of the neuromuscular blocking agents resulted in significant concentration differences ($p < 0.0001$ for

all NMBD pairs). To better assess the true affinity of SRBAs for muscle relaxants, we calculated the ratio of EC_{50} and EC_{90} to the concentration of each NMBD for each of the two reversal agents, thus obtaining the corrected effective concentration values ($EC_{50,kek}$, $EC_{90,kek}$). For rocuronium-induced muscle relaxation, the CMGCD $EC_{50,kek}$ was 3.81 μM (2.44–5.19 μM) and the $EC_{90,kek}$ was 10.60 μM (9.63–11.57 μM). For rocuronium-induced muscle relaxation, sugammadex $EC_{50,kek}$ was 0.34 μM (0.24–0.44 μM), $EC_{90,kek}$ was 0.55 μM (0.48–0.62 μM) and $EC_{99,kek}$ was 0.95 μM (0.82–1.08 μM). For relaxation induced by pipecuronium, the value of CMGCD $EC_{50,kek}$ was 5.15 μM (4.21–6.08 μM) and $EC_{90,kek}$ was 11.02 μM (8.95–13.08 μM). For pipecuronium-induced muscle relaxation, sugammadex $EC_{50,kek}$ was 0.38 μM (0.23–0.54 μM), $EC_{90,kek}$ was 0.58 μM (0.43–0.74 μM), and $EC_{99,kek}$ was 0.95 μM (0.69–1.20 μM). For vecuronium, we obtained that the value of CMGCD $ED_{50,kek}$ was 101.2 μM (73.9–128.4 μM), $EC_{90,kek}$ was 306.0 μM (172.7–439.2 μM). For vecuronium, sugammadex $EC_{50,kek}$ was 0.32 μM (0.23–0.41 μM), $EC_{90,kek}$ was 0.68 μM (0.54–0.83 μM), and $EC_{99,kek}$ was 1.67 μM (1.08–2.26 μM). For sugammadex, there was no statistically significant difference in the values of $EC_{50,kek}$ and $EC_{90,kek}$ for either pipecuronium, rocuronium or vecuronium. The concentration-response curves for sugammadex were very steep, hence the $EC_{99,kek}$ values were also calculated. The $EC_{99,kek}$ value for vecuronium was significantly higher than for pipecuronium ($p = 0.024$) and rocuronium ($p = 0.013$). For CMGCD, the $EC_{50,kek}$, and $EC_{90,kek}$ values were significantly higher for vecuronium compared to these values for rocuronium and pipecuronium. The $EC_{50,kek}$, and $EC_{90,kek}$ values for rocuronium were not significantly lower than those calculated for pipecuronium.

4.2. Examining the effect of magnesium

4.2.1. Effect of magnesium on neuromuscular transmission

As a first step, the magnesium concentration-effect curve was determined (see Figure). 4.06 mM (95% CI: 3.91–4.21 mM) was required to reach 50% of the effective concentration (EC_{50}). The concentration-effect curve shows that increasing the magnesium concentration from 1 mM to 1.5 mM decreased the ST force amplitude to an average of 96.6% (95% CI: 91.4–101.8%), compared to the amplitude measured at a magnesium concentration of 1 mM (Figure 5).

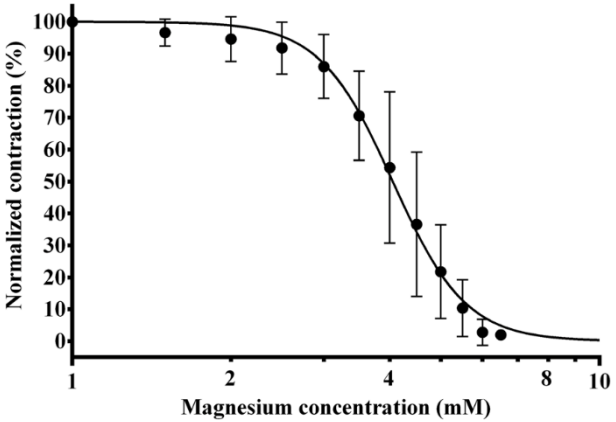

Figure 5. Magnesium concentration-response curve. Normalized contraction force amplitude plotted as a function of the decimal logarithm of the magnesium concentration (log_{10}). The curves are best-fit curves, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points.

4.2.2. Effect of magnesium on rocuronium-induced neuromuscular block

The concentration-response curves for rocuronium were also plotted at 1 mM and 1.5 mM magnesium concentrations. The EC_{50} value of rocuronium at 1 mM magnesium concentration was 7.5 μM (6.93–8.12 μM) and EC_{95} was 12.89 μM (10.67–15.56 μM). At 1.5 mM magnesium concentration, rocuronium EC_{50} was 4.26 μM (4.09–4.43 μM) and EC_{95} was 7.35 μM (6.64–8.13 μM) ($p < 0.0001$). Increasing magnesium concentration resulted in a leftward shift in the muscle relaxant concentration-response curve (Figure 6).

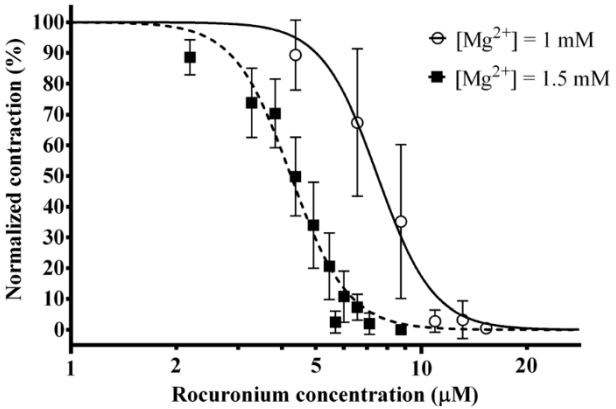

Figure 6. Rocuronium concentration-response curves at two different magnesium concentrations of 1 mM and 1.5 mM. Normalized contraction force amplitude plotted as a function of the logarithm of the decimal of the concentration of rocuronium (log_{10}). Curves are best-fit curves, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points.

4.2.3. Effects of magnesium on the reversibility of rocuronium-induced neuromuscular block by sugammadex

For this measurement, muscle relaxation was induced with rocuronium at a concentration of 1 mM and 1.5 mM magnesium, and then the concentration of Sugammadex in solution was increased stepwise to test for the suspension of neuromuscular block. The concentration-response curves of Sugammadex recorded at two different magnesium concentrations are shown in Figure 7. In our results, we obtained that the EC_{50} value of sugammadex at 1 mM magnesium concentration was 3.67 μM (3.43–3.92 μM) and at 1.5 mM magnesium concentration was 1.51 μM (1.42–1.50 μM). The effective concentration for the 95% muscle force contraction return (EC_{95}) was 7.22 μM (6.09–8.54 μM) at 1 mM magnesium concentration and 4.48 μM (3.80–5.29 μM) at 1.5 mM magnesium concentration. The concentration differences between EC_{50} and EC_{95} measured at 1 mM and 1.5 mM magnesium concentrations were statistically significant ($p < 0.0001$ for EC_{50} and $p = 0.002$ for EC_{95}).

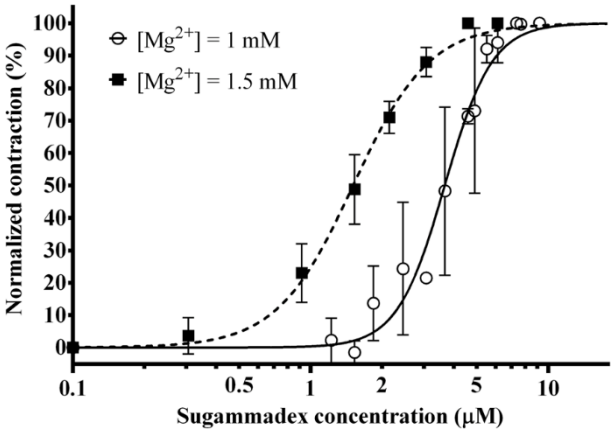

Figure 7. Sugammadex concentration-response curves during the reversal of rocuronium-induced muscle relaxation at two different magnesium concentra-

tions of 1 mM and 1.5 mM, elevated before muscle relaxation. Normalized contraction force amplitude plotted as a function of the logarithm of the decimal of the concentration of sugammadex (log_{10}). Due to the potentiating effect of the magnesium muscle block, the concentration of rocuronium was 0.3 mg at a magnesium concentration of 1.5 mM and 0.5 mg at a magnesium concentration of 1 mM. The curves are best-fit curves, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points.

Although the apparent potency of sugammadex appears to increase as the magnesium concentration is increased, the effect is confounded by the fact that a higher magnesium concentration at a lower rocuronium concentration induces the same degree of neuromuscular block. In our experiments, to achieve a 90% ST force amplitude reduction, 0.5 mg rocuronium was required at a magnesium concentration of 1 mM, and only 0.3 mg rocuronium was required at a magnesium concentration of 1.5 mM. In order to exclude the effects of different rocuronium concentrations on the reversal of muscle relaxation by sugammadex, we increased the magnesium concentration after partial reversal of neuromuscular block, thus achieving the same concentration of rocuronium to observe the effect of sugammadex at different magnesium concentrations (Figure 8). The increased magnesium concentration in this set-up, resulted in a steep decrease in ST force amplitude and a consequent increase in the EC_{50} value of sugammadex, i.e. from 3.67 μM (3.43–3.92 μM) to 5.36 μM (5.18–5.53 μM), resulting in a statistically significant difference ($p < 0.0001$). However, there was no statistically significant difference ($p = 0.542$) between the EC_{95} values, increasing from 7.22 μM (6.09–8.54 μM) to 7.61 μM (7.05–8.20 μM).

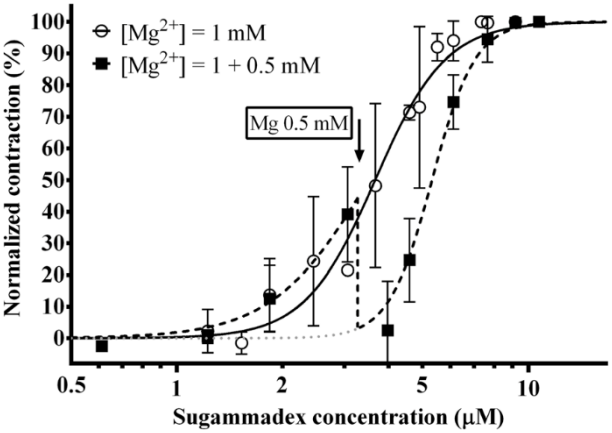

Figure 8. Effect of magnesium after muscle relaxation on the concentration-response curve of sugammadex during rocuronium-induced muscle relaxation. Normalized contraction force amplitude plotted as a function of the decimal logarithm of sugammadex concentration (log_{10}). At a magnesium concentration of 1 mM, 0.5 mg rocuronium was administered and reversed with sugammadex, either at the same magnesium concentration (○) or after partial reversion at a magnesium concentration increased to 1.5 mM (■). The magnesium concentration was increased after reversal of partial muscle relaxation, indicated by the black arrow, to visualize the contractions despite the potentiating effect of magnesium muscle block. The black dotted line **(---)** shows the actual normalized contraction force amplitude, while the grey dotted line (●) shows the best-fitting curve, approximated by non-linear regression, from n = 5 different preparations. Measured points represent the mean value of the normalized force amplitude at a given concentration. Error bars represent the standard deviation of the measurement points.

4.2.4. Effect of magnesium after reversal of neuromuscular block by sugammadex

Since the addition of magnesium to the solution after reversal with partial sugammadex resulted in a decrease in ST-force amplitude and an increase in the slope of the concentration-response curve, we wanted to investigate whether the traditional measurement of safe neuromuscular reversal remains reliable under varying magnesium concentrations. Therefore, neuromus-

cular block was reversed either completely (TOF ratio ≈ 1, mean TOF ratio 0.99 ± 0.006) or above the safety limit but with a still visible decrease (TOF ratio > 0.9, mean TOF ratio 0.95 ± 0.02), and magnesium concentration in solution was subsequently increased. The sugammadex/rocuronium ratio was 1.43 (0.83–2.02) for TOF ratio ≈ 1 group and 0.68 (0.60–0.76) for TOF ratio > 0.9 group. While the TOF ratio remained stable with increasing magnesium concentration in the TOF ratio ≈ 1 group, a steady decrease in the TOF ratio was observed in the TOF ratio > 0.9 group, and a return of neuromuscular block below the safety level was observed (Figure 9).

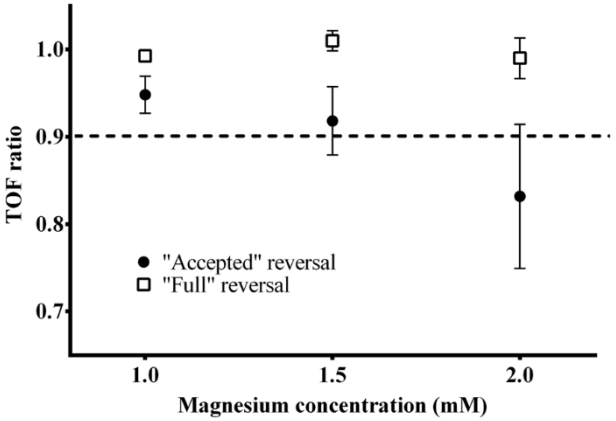

Figure 9. Magnesium-induced change in the train-of-four (TOF) rate. TOF rate as a function of magnesium concentration. After a 0.5 mg rocuronium concentration, sugammadex was administered until the clinically accepted value of TOF rate was reached, i.e. 1 > TOF rate > 0.9, but fatigue is still visible, or until complete suspension, i.e. TOF rate ≈ 1.0, fatigue is not visible. Subsequently, the magnesium concentration was increased to 1.5 mM and then to 2.0 mM. Each type of suspension was measured from n = 5 different preparations. Measured points represent the average TOF rate at a given concentration. Error bars represent the standard deviation of the measurement points.

5. Discussion

Muscle relaxation, which is one of the mainstays of general anaesthesia, can cause serious complications if its effects persist after extubation. Because of the postoperative residual neuromuscular block and the side effects caused by acetylcholinesterase inhibitors, clinical practice has favoured the use of neuromuscular blocking agents with intermediate duration of action over long-acting muscle relaxants. Nevertheless, PORNB has not been reliably eliminated even with the use of intermediate-duration muscle relaxants, and therefore routine monitoring of neuromuscular block and its appropriate reversal, if necessary, are essential for adequate patient safety. The use of selective relaxant binding agents, notably sugammadex, results in rapid and safe reversal of muscle relaxants with few side effects. However, allergic reactions and anaphylactic shock have been described rarely with the use of this compound in both adults and children. In addition, very rare, severe atropine-resistant bradycardia and asystole have been reported in studies. In addition, sugammadex is ineffective against benzylisoquinoline-based muscle relaxants such as atracurium or cisatracurium. For these reasons, a constant search is underway for alternative antagonists of neuromuscular blocking agents with a broader spectrum of reversal and fewer side effects.

In our experiments, we investigated the effect of a new selective muscle relaxant binding compound, carboxymethyl-γ-cyclodextrin, on the ability to revers muscle relaxation induced by rocuronium, vecuronium, and pipecuronium, and compared it with sugammadexes, which is already in clinical use. To investigate the efficacy of γ-cyclodextrin derivatives on the reversal of neuromuscular block, we used in our experiments an isolated tissue preparation consisting of the hemidiaphragm of a rat and the associated pherenic nerve. This hemidiaphragm-nervus phrenicus preparation, originally described by Bülbring, has been

used in many classical experiments in the search for the pharmacology of neuromuscular junction. Since the normal function of neuromuscular junction and the electromechanical transmission following nerve stimulation is preserved in the hemidiaphragm-nervus phrenicus preparation, this ex vivo system is suitable for the investigation of clinically relevant data without the need for live animal experiments.

In our experiments, we first constructed concentration-response curves to determine the EC_{50} of the three aminosteroid muscle relaxants used in clinical practice, namely pipecuronium, vecuronium and rocuronium. The EC_{50} values we obtained were similar to previously published measurement results measured in a similar system, thus supporting the validity of our results.

Our results showed that pipecuronium had the highest efficacy of the three muscle relaxants, followed by vecuronium and then rocuronium.

After plotting the concentration-response curves of muscle relaxants, the efficacy of two γ-cyclodextrin derivatives, sugammadex and carboxymethyl-γ-cyclodextrin, on relaxant reversibility was investigated. Our results showed that both sugammadex and CMGCD were able to antagonize the neuromuscular block produced by both pipecuronium, vecuronium and rocuronium, but carboxymethyl-γ-cyclodextrin with a less efficacy.

Sugammadex, which was originally developed to revers rocuronium-induced neuromuscular block, has already been shown by several experiments to be capable of reversing both vecuronium- and pipecuronium-induced muscle relaxation, which our results have confirmed. Results from in vitro and in vivo experiments are available to establish the efficacy of the reversal agent against various muscle relaxants. The results of an in vitro experiment showed that while the rocuronium association constant of sugammadex is 1.79×10^7 mol/L, the vecuronium association constant of sugammadex is only 5.72×10^6 mol/L, which implies that the formation of rocuronium complex with sugam-

madex is faster than the formation of vecuronium complex with the reversal agent. The dissociation constant of the sugammadex-vecuronium complex is 0.17 μM, which is 3.1 times higher than the dissociation constant of rocuronium sugammadex, which is only 0.055 μM. This results in the complex formation of sugammadex with vecuronium being not only slower, but also requiring a higher relative concentration of the reversal agent than that of rocuronium. In vivo experiments with both sugammadex and vecuronium show that the relaxant binding agent is less effective with vecuronium than with rocuronium. In vitro experiments to assess the affinity of sugammadex and pipecuronium have not yet been performed, but clinical in vivo experiments show that the affinity of the reversal agent for pipecuronium is similar to that for rocuronium. Our results also demonstrate this, i.e. sugammadex showed similar affinity for pipecuronium, rocuronium and vecuronium when looking at concentration corrected EC_{50} and EC_{90} and decreased affinity for vecuronium at corrected EC_{99}. The ratio of the EC_{99} value to the concentration of pipecuronium and rocuronium was approximately 1, indicating that equimolar concentrations of sugammadex were sufficient to fully recover muscle contraction during the reversal of the neuromuscular block produced by these two muscle relaxants. For vecuronium, this ratio was close to 1.7, which meant that the binding capacity was reduced, i.e. almost twice as many molecules of sugammadex were needed to bind all the vecuronium.

The results of our experiments with the carboxymethyl-γ-cyclodextrin derivative showed that the compound was able to antagonize the neuromuscular block induced by both pipecuronium, vecuronium and rocuronium. There was no statistically significant difference in the concentration corrected EC_{50} and EC_{90} values in case os rocuronium and pipecuronium, although it showed grater affinity for pipecuronium. For vecuronium, the CMGCD potential was low, with comparably higher EC_{50} and EC_{90} values, even after concentration correction. The efficacy of CMGCD to revers muscle relaxation was different for different

muscle relaxants, as supported by the increased contraction force amplitude that occurred after the addition of sugammadex for rocuronium and vecuronium.

When comparing the muscle relaxant inhibitory efficacy of the two γ-cyclodextrin derivatives, we found that CMGCD reversed the muscle block produced by rocuronium and pipecuronium, but with an efficacy about 15–20 times lower than that of sugammadex, which translated into clinical practice would mean that the drug would have to be used at a dose this much higher to achieve comparable effects to sugammadex.

The limitation of our experiments arises from their ex vivo nature. The pharmacokinetic effects of the compounds are eliminated in our experiments, so there may be a difference in actual muscle block dynamics in vivo. We used ST stimulation to monitor neuromuscular function, which is less sensitive than the TOF ratio used in the clinical practice. Furthermore, the relationship between fading in TOF ratio and ST depression may be drug specific. Finally, the concentration-response values measured in vitro may not reflect some of the variables measured in clinical studies, such as the time to return to muscle strength or the onset of recurrence.

In our series of experiments on magnesium, we investigated the neuromuscular effects of magnesium and its interaction with rocuronium and sugammadex. Consistent with previous studies, we found that magnesium alone reduced muscle contraction in a concentration dependent manner. The multifactorial effects of magnesium on neuromuscular function have been known for decades. The most prominent of these is the reduction of acetylcholine release on prejunctionally located motor neurons. Of lesser importance are the postjunctional effects of magnesium, including reduction of depolarisation at the motor endplate and reduction of muscle fibre membrane excitability. These effects of magnesium can be reversed by increasing calcium concentrations, which implies a role for magnesium as a calcium antagonist. The introduction of the use of parenteral magnesium in the

clinical setting rapidly demonstrated the potentiating effect of the ion on neuromuscular agents. In 1970, the concentration-effect relationship of the combined use of magnesium and muscle relaxants was studied in a rat phrenic nerve-hemidiaphragm preparation. Their results showed that the addition of 0.1 mg/mL of magnesium to the solution prior to the application of the musclerelaxant caused a leftward shift in the concentration-effect curve of tubocurarine, demonstrating a potentiating effect of the ion on the muscle relaxant applied, which their results showed to be 4.1-fold (3.9–4.2). In 2018, also in an experiment on a rat phrenic nerve-hemidiaphragm preparation, the concentration-response curves of rocuronium-induced muscle relaxation were measured at four different magnesium concentrations, 1 mmol/L, 2 mmol/L, 3 mmol/L and 4 mmol/L. Their results showed that the EC_{50} , EC_{90} and EC_{95} of rocuronium decreased significantly as the magnesium concentration increased, except at concentrations of 3 and 4 mmol/L. The result of our experiment, also performed on the rat phrenic nerve-diaphragm preparation, is also that magnesium caused a leftward shift in the muscle relaxant concentration-response curve, similar to that seen previously. A synergistic relationship between rocuronium and magnesium can be assumed, since when they are used together in solution, the decrease in muscle force contraction is found to exceed the sum of the decrease in muscle force contractions induced by rocuronium and magnesium separately. It was previously hypothesized that this potentiation is a consequence of the inhibitory effect of magnesium on the release of acetylcholine, which the ion induces through inhibition of presynaptic voltage-dependent calcium channels. But in 2011, an in vitro experiment was conducted where, measured by a patch clamp technique, it was found that the enhancement of the clinical effect of the muscle relaxant by magnesium is not only the result of the ion's presynaptically exerted action, but is partly due to the synergistic action of the ion and the relaxant on the nicotinic ACh receptor itself.

In our experiments, the pre-block applied magnesium also seems to increase the effectiveness of sugammadex. This is mainly due to the fact that, at the higher magnesium concentration less rocuronium was needed to achieve the same level of neuromuscular block due to the potentiating effect of magnesium on rocuronium. It follows that the amount of rocuronium that sugammadex has to bind is less, which caused the apparent leftward shift in the concentration-effect curve of sugammadex. In contrast, when the magnesium concentration was increased only after the onset of neuromuscular block reversal, i.e., at partial reversal, the potentiation of rocuronium at the increased magnesium concentration induced muscle relaxation and resulted in a rightward shift in the concentration-response curve of sugammadex. It should be noted that even under these conditions, we did not see a significant increase in the total reversal concentration of sugammadex. Although the possibility that there is no interaction between magnesium and sugammadex cannot be completely ruled out on the basis of our experimental data, a microcalorimetric study demonstrated that there is no affinity between magnesium and sugammadex. Hence, the possibility of a clinically significant interaction between magnesium and sugammadex is very small and probably irrelevant compared to the more pronounced potentiating effect of magnesium on rocuronium.

In 1996, an in vivo experiment with vecuronium was performed in 20 patients, demonstrating that 60 mg/kg magnesium administered upon return of muscle strength to TOFR 0.7 caused recurarisation in all patients, with muscle paralysis persisting for at least one hour. The recurarisation was rapid in onset and of such magnitude that it had a negative effect on respiration. The rapid and large onset of recurarisation was explained by the "margin of safety" concept of neuromuscular transmission, according to which, despite a satisfactory muscle response to electrical stimulation, 70–80% of ACh receptors may still be blocked by muscle relaxants, so that magnesium, which causes inhibition of ACh release, may lead to recurarisation. A similar experience was shared in a case study in which a patient had muscle relaxation

induced with rocuronium reversed by sugammadex and then administered a high concentration of magnesium due to the onset of atrial fibrillation. In our measurements, we found that the effect of magnesium persists even at TOF ratios > 0.9, which clinically already fulfils the condition of safe extubation, and that only complete reversal of neuromuscular block with sugammadex prevents the return of neuromuscular block by magnesium. The magnesium-induced neuromuscular block may be of such magnitude that the level of block returns to the "no longer safe for extubation" zone (TOF ratio < 0.9). The change in magnesium concentration that can already induce the return of muscle relaxation to the extent described above is relatively small, since the highest magnesium concentration used in our measurements was 2 mM, which is close to the physiological range of the ion. The concentration ratio of sugammadex to rocuronium indicates that there are extra sugammadex molecules in solution in complete reversal, taking into account the 1:1 binding of sugammadex to rocuronium, i.e. all rocuronium is encapsulated by sugammadex. In contrast, if the neuromuscular block is only reversed up to a TOF ratio > 0.9, a significant fraction of rocuronium will not be encapsulated by sugammadex. Our results show that at this level of neuromuscular block, 25–40% of rocuronium molecules are not bound to sugammadex, which is consistent with other studies in mice. It is likely that the potentiation of these unbound rocuronium molecules is responsible for the decrease in TOF rate following magnesium administration.

Due to the ex vivo nature of our experiments, we cannot predict the applicability of our results in clinical practice. One of the most confounding factors is that the tissue preparation spends quite a long time in a hypoxic state during the preparation process, and it is not yet clear what receptor level changes this might lead to, or to what extent it might affect intracellular ion flux and utilisation, although there have been attempts to explore this. However, if there is any such effect, it is in turn equal across the experimental specimens as they were used in the same way.

However, the distinct advantage of this ex vivo approach is that it provides accurate quantitative results that would be much more difficult to determine in vivo. Relationships and interactions are revealed that are otherwise masked by the use of drugs at high concentrations in clinical trials (relative to the concentrations needed to define concentration-effect curves), or to study endpoints that are surrogates for real efficacy (time to reversal of neuromuscular block by sugammadex versus a real concentration-effect relationship). When interpreting the existing clinical results in our ex vivo experiments, several clinically relevant hypotheses regarding the effect of magnesium can be made.

When magnesium is administered prior to the administration of a neuromuscular blocking agent and there is no compensatory reduction in the concentration of the muscle relaxant administered, the potentiating effect and the leftward shift of the concentration-effect curve results in a faster onset of action and a longer neuromuscular blocking effect, an effect that has been previously demonstrated with vecuronium and rocuronium. While one might hypothesise that the potentiation of neuromuscular block caused by magnesium pretreatment would prolong the reversal of muscle relaxation by sugammadex, two clinical studies have shown that magnesium has no significant effect on it. This was confirmed by a 2018 ex vivo rat phrenic nerve-hemidiaphragm preparation experiment, which showed that the average time from sugammadex administration to the appearance of the maximal first twitch under TOF stimulation, was not significantly longer at a magnesium concentration of 1 mmol/L (1039 ± 351.8 seconds) and 2 mmol/L (926 ± 278.1 seconds), only at the higher magnesium concentration of 4 mmol/L (1483.9 ± 237 seconds).

Administration of magnesium after incomplete muscle force recovery, spontaneously or with the use of a reversal agent, can cause recurarisation, even if TOFR > 0.9. Two case reports and an in vivo experiment have been described, drawing clinicians' attention to the recurarisation effect of magnesium. The degree

of recurarisation may already be clinically significant at magnesium concentrations that are still close to the physiological range. For this reason, the postoperative use of magnesium as an adjuvant analgesic agent, which is now becoming more widespread, requires great care and quantitative monitoring of neuromuscular function is recommended to ensure a full return of muscle strength to prevent recurarasation, even after reversal with sugammadex.

6. Own results, new findings

1. We studied for the first time in the international literature, the effect of carboxymethyl-γ-cyclodextrin DS = 4,1 on the reversal of aminosteroid type muscle relaxants.
2. Carboxymethyl-γ-cyclodextrin DS = 4.1 effectively inhibited rocuronium and pipecuronium-induced muscle relaxation in this ex vivo system.
3. The carboxymethyl-γ-cyclodextrin DS = 4.1 inhibited rocuronium- and pipecuronium-induced muscle block, but with an efficiency about 15–20 times lower than that of sugammadex. This difference in efficiency could be compensated by increasing the concentration of carboxymethyl-γ-cyclodextrin. By optimising the degree of substitution of carboxymethyl-γ-cyclodextrin, the affinity of the reversal agent could possibly be improved and thus its concentration reduced.
4. Magnesium markedly increases the rocuronium-induced muscle relaxation in the ex vivo system and results in a leftward shift in the muscle relaxant concentration-response curve.
5. We consider it an important new observation that the use of magnesium reduces the safety margin of the reversal due to the rocuronium molecules which are not bound by sugammadex. This phenomenon can only be eliminated by the full reversal of rocuronium with sugammadex. Our observations highlight the importance of patient safety in the postoperative period and the need for postoperative neuromuscular monitoring when magnesium and muscle relaxant are used together.

7. Summary

The incidence of postoperative residual neuromuscular block is still high and can have serious consequences for patients. Over the decades, many innovations have appeared to prevent it, such as medium-acting muscle relaxants, quantitative monitoring devices, selective relaxant binding agents, but even these have failed to reduce its incidence. With the introduction of the γ-cyclodextrin derivative sugammadex, rapid reversal of neuromuscular block of any depth was possible. But this compound is also far from being the ideal muscle relaxant reversal agent. Among other things, it can only reverse the aminosteroid type muscle relaxant, and has been described as having serious side effects when used, as well as being very expensive. Ex vivo, using rat phrenic nerve-diaphragm preparations, we have also investigated the efficacy of a new muscle relaxant binding agent. The compound we used is also a γ-cyclodextrin derivative, carboxymethyl-γ-cyclodextrin, with a substitution degree of 4.1. The results of our experiments showed that although carboxymethyl-γ-cyclodextrin is also effective in inhibiting rocuronium and pipecuronium-induced muscle relaxation, sugammadex is 15–20 times more effective in inhibiting the effects of these muscle-blocking compounds. It would be advisable to optimize the degree of substitution of carboxymethyl-γ-cyclodextrin to improve the affinity of the compound for muscle relaxants and to reduce its concentration. The efficacy, predictability and reversibility of muscle relaxants are influenced by several factors, all of which contribute to the increased risk of postoperative residual neuromuscular block. One of these influencing factors is magnesium. In our second series of experiments, we investigated the effect of this ion on rocuronium-induced muscle relaxation and its reversibility in the ex vivo rat phrenic nerve-diaphragm preparation. Magnesium increased the degree of rocuronium-induced muscle relaxation in a dose dependent manner. When we examined how

increased magnesium concentration affected the ability of sugammadex to reverse rocuronium-induced muscle relaxation, we found that increasing the magnesium concentration from 1.0 mM to 1.5 mM did not significantly affect the concentration of sugammadex required for complete reversal. Magnesium administered after rocuronium-induced muscle relaxation reversed by sugammadex to a TOFR > 0.9 caused recurarization even at a magnesium level of 2 mM. The magnesium-induced reparalysis was prevented only by the complete reversal of the muscle block. Magnesium administered after reversal of muscle relaxation requires increased attention by clinicians and quantitative monitoring of muscle function is essential to ensure adequate inhibition of muscle relaxants to prevent postoperative residual neuromuscular block.

8. Publication list

DEBRECENI EGYETEM

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

Nyilvántartási szám: DEENK/320/2023.PL
Tárgy: PhD Publikációs Lista

Jelölt: Csernoch Vera
Doktori Iskola: Idegtudományi Doktori Iskola

A PhD értekezés alapjául szolgáló közlemények

1. Fábián, Á. I., Tassonyi, E., **Csernoch, V.**, Fedor, M., Sohajda, T., Szente, L., Fülesdi, B.: Carboxymethyl-γ-cyclodextrin, a novel selective relaxant binding agent for the reversal of neuromuscular block induced by aminosteroid neuromuscular blockers: an ex vivo laboratory study.
BMC Anesthesiol. 21 (1), 1-9, 2021.
DOI: http://dx.doi.org/10.1186/s12871-021-01424-4
IF: 2.376
2. Fábián, Á. I., **Csernoch, V.**, Tassonyi, E., Fedor, M., Fülesdi, B.: The effect of magnesium on the reversal of rocuronium-induced neuromuscular block with sugammadex: an ex vivo laboratory study.
BMC Anesthesiol. 19 (64), 1-8, 2019.
DOI: http://dx.doi.org/10.1186/s12871-019-0734-6
IF: 1.695

A közlő folyóiratok összesített impakt faktora: 4,071
A közlő folyóiratok összesített impakt faktora (az értekezés alapjául szolgáló közleményekre): 4,071

A DEENK a Jelölt által az iDEa Tudóstérbe feltöltött adatok bibliográfiai és tudománymetriai ellenőrzését a tudományos adatbázisok és a Journal Citation Reports Impact Factor lista alapján elvégezte.

Debrecen, 2023.07.03.

9. Keywords

Phrenic nerve-diaphragm preparation, Neuromuscular block, Rocuronium, Pipecuronium, Vecuronium, Carboxymethyl-γ-cyclodextrin, Sugammadex, Magnesium

10. Acknowledgements

First of all, I would like to thank my supervisor, Prof. Dr. Béla Fülesdi for his trust, support and help in my work.

I owe my thanks to my co-supervisor Dr. István Ákos Fábián for his help and work both in the experiments and in the writing of the papers.

I owe a debt of gratitude to Prof. Dr. Edömér Tassonyi, whose decades of experience have been a great help in designing and setting up the studies.

I would like to thank Lajos Szente and Tamás Sohajda of Cyclolab Ltd for the preparation of the compounds and their contribution to the experiments.

Thank you to Dr. Marianna Fedor for her assistance in the examinations.

Many thanks to the Head of the Department of Clinical Physiology Prof. Dr. Zoltán Papp and his colleague Dr. Tamás Csípő for allowing us to carry out our experiments and for their help in the handling of the experimental animals.

Last but not least, I would like to thank my family and friends for their patience, support and encouragement over the years.