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The effects of varying Mg^{2+} ion concentrations on contractions to the cotransmitters ATP and noradrenaline in the rat vas deferens

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NF449 octasodium salt (PubChem CID: 6093161)
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ABSTRACT

The vas deferens responds to a single electrical pulse with a biphasic contraction caused by cotransmitters ATP and noradrenaline. Removing Mg^{2+} (normally 1.2 mM) from the physiological salt solution (PSS) enhances the contraction. This study aimed to determine the effect of Mg^{2+} concentration on nerve cotransmitter-mediated contractions. Rat vasa deferentia were sequentially bathed in increasing (0, 1.2, 3 mM) or decreasing (3, 1.2, 0 mM) Mg^{2+} concentrations. At each concentration a single field pulse was applied, and the biphasic contraction recorded. Contractions to exogenous noradrenaline 10 μM and ATP 100 μM were also determined. The biphasic nerve-mediated contraction was elicited by ATP and noradrenaline as NF449 (10 μM) and prazosin (100 nM) completely prevented the respective peaks. Taking the contractions in normal PSS (Mg^{2+} 1.2 mM) as 100%, lowering Mg^{2+} to 0 mM enhanced the ATP peak to 170 ± 7% and raising Mg^{2+} to 3 mM decreased it to 39 ± 3%; the noradrenaline peak was not affected by lowering Mg^{2+} to 0 mM (97 ± 3%) but was decreased to 63 ± 4% in high Mg^{2+} (3 mM). Contractions to exogenous ATP, but not noradrenaline, were increased in Mg^{2+} 0 mM and both were inhibited with Mg^{2+} 3 mM. Changing Mg^{2+} concentration affects the contractions elicited by the cotransmitters ATP and noradrenaline. The greatest effects were to potentiate the contraction to ATP in Mg^{2+} 0 mM and to inhibit the contraction to both ATP and noradrenaline in high Mg^{2+}. Future publications should clearly justify any decision to vary the magnesium concentration from normal (1.2 mM) values.

1. Introduction

Mg^{2+}, being the second most abundant intracellular cation after K^+, plays an important role in many physiological functions by regulating both cell and tissue functions (Jahnen-Dechent and Ketteler, 2012; Laires et al., 2004). The composition of normal Krebs' physiological salt solution (PSS) includes Mg^{2+} 1.2 mM while human serum Mg^{2+} concentrations are between 0.65 and 1.05 mM (Jahnen-Dechent and Ketteler, 2012). The electrically stimulated vas deferens has been used as a bioassay in studies of a range of pharmacological and physiological processes, ever since its introduction by Hukovic in 1961. Hukovic (1961) showed that the densely innervated tissue responds to electrical stimulation of the hypogastric nerve with longitudinal contractions. More specifically, nerve stimulation of the vas deferens induces the release of the cotransmitters noradrenaline and ATP from postganglionic sympathetic nerves, causing a biphasic contraction (reviewed in more detail by Burnstock and Verkhratsky (2010) and Burnstock (2014). ATP release elicits a fast contraction (phase 1) by acting on postjunctional P2X1 ion channel receptors, while noradrenaline release causes slower contraction (phase 2) by acting on postjunctional α_{1A}-adrenoceptors (Burnstock, 1972; Mallard et al., 1992; McGrath, 1978; Westfall et al., 1978).

Many studies using the isolated vas deferens bioassay have been performed in modified Krebs-Henseleit solution without Mg^{2+} (Berzetei-Gurske et al., 1996; Christopoulos et al., 2001; Corbett et al., 1984; Hourani et al., 1993; Lay et al., 2000; North and Surprenant, 2000; Pertwee et al., 2002; Pertwee et al., 1992; Wiley et al., 2011). Some reports have indicated that the reason for the use of Mg^{2+}-free Krebs' PSS was to enhance the response to nerve stimulation (Hourani et al., 1993). The idea of excluding Mg^{2+} from the Krebs' PSS may have

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originated from a study by Hughes et al. (1975) investigating the effect of morphine on adrenergic transmission in the mouse vas deferens. They observed that 1.4 mM of Mg^{2+} caused a 40–60% decrease in contractions of the vas deferens compared to contractions in Mg^{2+}-free solution.

Elucidating the effect of varying Mg^{2+} concentrations on neurotransmitter-induced contractions in the vas deferens may allow for a standardisation of the bioassay. In this study, we used just a single electrical field pulse, repeated every 30 min, rather than dual field pulses (Ellis and Burnstock, 1990) or trains of stimuli to prevent modulation of the release of the cotransmitter agents (prejunctional) by other neuromodulators or actions at the postsynaptic receptors (Driessen et al., 1994; French and Scott, 1983). In addition, we compared the results from the single field pulse with the contractions of single high concentrations of exogenous ATP, noradrenaline and methoxamine in the presence of Mg^{2+} 0, 1.2 and 3 mM. To test whether Mg^{2+} concentrations were directly affecting the release of transmitters ATP and noradrenaline, we determined the IC_{50} for the highly selective prejunctional N-type calcium channel ($Ca_v2.2$) inhibitor ω-conotoxin GVIA on the contraction to a single pulse.

Our results show that ATP-mediated contractions are enhanced by the exclusion of Mg^{2+} compared with normal Mg^{2+} concentration (1.2 mM), while contractions mediated by both cotransmitters, ATP and noradrenaline, are inhibited by high Mg^{2+} (3 mM). These findings may be helpful to future researchers in designing protocols to investigate pre- or post-junctional modulation of these cotransmitters in sympathetic transmission.

2. Materials and methods

The Ethics Committee of the University of Melbourne approved experiments in accordance with The Australian Code for the care and use of animals for scientific purposes (8th edition, 2013, National Health and Medical Research Council, Canberra).

2.1. Tissue collection and set up

Male Sprague-Dawley rats (250–350 g) were deeply anaesthetised by inhalation of 5% isoflurane in oxygen and killed by a rapid cut through the spinal cord. The whole vasa deferentia were excised and placed in modified Krebs-Henseleit PSS with the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, EDTA 0.026, $MgSO_4$ 0, 1.2 or 3 ($MgSO_4$ content was dependent on the protocol), oxygenated with 95% O_2 and 5% CO_2 at pH 7.4.

The tissues were pinned down and tied at either side with a silk thread and trimmed to an approximate length of 2 cm. The prostatic end was tied to a stainless-steel hook on a fixed acrylic organ bath leg between two parallel platinum field electrodes, while the epididymal end was tied uppermost to a stainless-steel hook attached to a Grass FTO3C isometric force transducer (Grass Instruments, Quincy, MA, USA) connected to a bridge amplifier and a data acquisition system, Powerlab 8/35 (ADInstruments, Sydney, Australia). The organ bath leg was adjusted vertically with an attached micrometer (Mitutoyo Manufacturing Co., Kawasaki, Japan). The responses were measured on a computer running LabChart 7 Pro software (ADInstruments) with the sampling rate: 100 Hz, range: 2 mV and low pass filter set at 20 Hz. Tissues were suspended in 5 ml organ baths containing either Mg^{2+}-free or 3 mM Mg^{2+} PSS, oxygenated with 95% O_2 and 5% CO_2 at 37 °C. The tissues were stretched to 2 g force, followed by a re-stretch to 2 g after 10 min. The bath solutions were changed twice before incubating the tissues with the appropriate PSS for 30 min.

2.2. Electrically stimulated vasa deferentia

Following incubation of the tissues in either Mg^{2+}-free or 3 mM Mg^{2+} PSS, a single square wave nerve stimulation pulse (150 V, 0.5 ms duration) was delivered by a Grass S88 stimulator *via* a Grass stimulus isolation unit (SIU5), which induced a biphasic contraction. This response was completely inhibited by pre-treatment with tetrodotoxin (0.1 μM) indicating that the electrical field pulse only depolarised the nerves and not the muscle. The tissues were incubated in PSS with 3 different concentrations of Mg^{2+} (0, 1.2 and 3 mM), whereby single pulse stimulations were induced at 30 min intervals, followed by 4 changes of the bath solution, then the composition of the PSS was changed from low to high Mg^{2+} content, or high to low Mg^{2+} content.

Experiments in Mg^{2+}-free PSS were also performed in the presence of the P2X1-purinoceptor antagonist NF449 (10 μM) (Braun et al., 2001) or the α_1-adrenoceptor antagonist prazosin (100 nM) (Cavero and Roach, 1980) or vehicle (MilliQ water, 5 μl). In separate experiments, the effects of varying the Mg^{2+} concentration on the sensitivity to ω-conotoxin GVIA, a potent and highly selective, inhibitor of the prejunctional N-type ($Ca_v2.2$) calcium channel (Pruneau and Angus, 1990; Whorlow et al., 1996), were tested by constructing ω-conotoxin GVIA concentration-response curves in each of the 3 Mg^{2+} concentrations.

The vasa deferentia were incubated with an antagonist or vehicle before inducing single pulse stimulations at 30 min intervals. Tissues were randomised into treatment groups.

2.3. Exogenous agonist stimulation of vasa deferentia with ATP, noradrenaline or methoxamine

The vasa deferentia tissues were contracted with either exogenous ATP 100 μM, noradrenaline 10 μM or methoxamine 10 μM following the same protocol as for the electrically stimulated tissues, with 4 changes of bath solution once the tissues had reached a maximum contraction (~10 s for ATP and ~30 s for noradrenaline or methoxamine). Tissues were randomised into treatment groups.

2.4. Drugs

Drugs used were: Adenosine 5′triphosphate (ATP) magnesium salt; methoxamine hydrochloride; noradrenaline bitartrate salt; prazosin hydrochloride; tetrodotoxin (all from Sigma-Aldrich, St Louis, MO, USA); NF449 octasodium salt (4,4′,4″,4‴-[carbonylbis[imino-5,1,3-benzenetriylbis(carbonylimino)]]tetrakis-1,3-benzenedisulfonic acid, octasodium salt; Cayman Chemical, Ann Arbor, MI, USA); and ω-conotoxin GVIA (Tocris Bioscience, Bio-Techne Ltd., Abingdon, United Kingdom). All drugs were dissolved in MilliQ water. Single-use aliquots of ATP (10^{-2} M), methoxamine (10^{-1} M), NF449 (10^{-2} M), noradrenaline (10^{-1} M), prazosin (10^{-3} M), tetrodotoxin (10^{-4} M) and ω-conotoxin GVIA (10^{-3} M) were stored at −20 °C.

2.5. Data and statistical analyses

All data are expressed as the mean ± S.E.M. from *n* experiments. *n* is the number of tissues from separate rats. The responses to the different concentrations of Mg^{2+} in the graphs are given either as the absolute increase from baseline tone or in percentage in relation to the contraction (100%) in normal PSS (Mg^{2+} 1.2 mM). Data were plotted and analysed using Prism 8 (Graphpad Software, La Jolla, CA, USA). Each individual ω-conotoxin GVIA concentration-response curve was fitted to a logistic sigmoidal 4 parameter model using Prism 8 to determine the Emax (initial force) and IC_{50} (concentration required to inhibit the contractions by 50%); these values were then averaged for each of the three Mg^{2+}-PSS conditions. For the comparison of the changes in the contractions between the three different PSS conditions, or for the comparison of the contractions between tissues in the presence of NF449, prazosin or vehicle, repeated measures one-way ANOVA with Dunnett *post hoc* test for multiple comparisons was performed. *P* values ≤ 0.05 were considered significant.

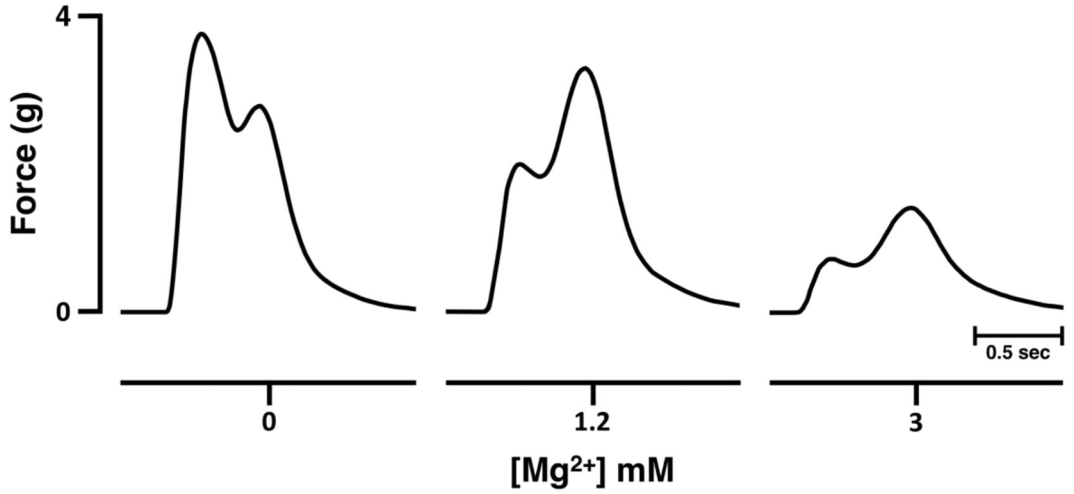

Fig. 1. Representative Labchart® traces of biphasic contractile responses of rat isolated vas deferens following a single electrical field pulse in the presence of PSS with different Mg^{2+} concentrations (0, 1.2 or 3 mM). In each case, the initial fast peak contraction is the ATP-mediated contraction while the second slow peak is the noradrenaline-mediated contraction.

3. Results

3.1. Do peak contractions to cotransmitters ATP and noradrenaline, phase 1 and 2 respectively, interact?

The high-speed digital recording of the contractile response to a single electrical field pulse clearly indicates two peaks or phases (Fig. 1). The first peak is considered to be the fast contraction to co-transmitter ATP and the second is the response to cotransmitter noradrenaline. As the Mg^{2+} is decreased to 0 mM the first peak is enhanced, but in high Mg^{2+} 3 mM, both peaks are attenuated compared with control Mg^{2+} 1.2 mM.

To confirm that the first and second peaks were mediated by ATP and noradrenaline, respectively, and whether there is any interaction between the cotransmitters, the contraction profile to a single field pulse was recorded in the absence of Mg^{2+} (0 mM), in drug-free PSS, or with prazosin 100 nM or NF449 10 μM to inhibit the post-junctional α_1-adrenoceptors or P2X1 purinoceptors, respectively. The competitive antagonism of either receptor type did not significantly alter the peak contraction response to the remaining cotransmitter compared to respective controls ($P > 0.2$, 1-way ANOVA; Fig. 2). The concentrations of prazosin and NF449 chosen to antagonise noradrenaline and ATP were sufficient to completely block the contraction peak to that co-transmitter (Fig. 2).

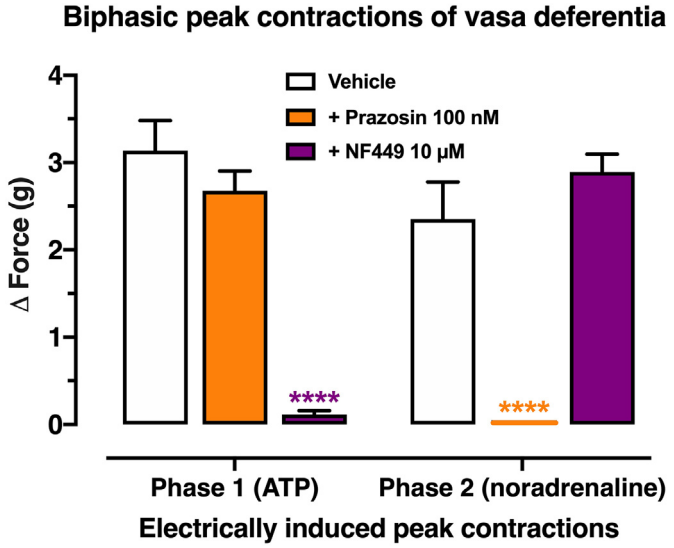

Fig. 2. Cotransmitter biphasic peak contractions of vasa deferentia in response to a single field pulse in Mg^{2+}-free PSS under 3 treatment conditions: (i) vehicle, open bars, $n = 5$; (ii) prazosin 100 nM, orange bars, $n = 6$; or (iii) NF449 10 μM, magenta bars, $n = 5$. The phase 1 peak was mediated by ATP acting on P2X1-receptors as it was sensitive to NF449 and the phase 2 peak was mediated by noradrenaline as it was abolished by prazosin. The results are shown under each condition after 150 min of treatment. The error bars are ± S.E.M. *n*, number of tissues from separate rats. One-way ANOVA with Dunnett *post hoc* test was performed; ****$P \leq 0.0001$ compared to respective vehicle group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Effects of Mg^{2+} on the biphasic contraction to a single electrical pulse

Since the distinct peaks in the contractile response to a single electrical pulse were separated in time and appeared to be independent of the other in magnitude, the time to reach peak 1 and peak 2 and the peak force were determined in the rising Mg^{2+} concentration protocol of 0, 1.2 and 3 mM and falling Mg^{2+} concentration protocol of 3, 1.2 and 0 mM. Time from the electrical stimulus to the first (ATP) peak was 279 ± 7 ms ($n = 6$), 261 ± 5 ms ($n = 6$) and 248 ± 3 ms ($n = 6$) for the average of both the rising and falling Mg^{2+} concentration protocols of 0, 1.2 and 3 mM (Fig. 3A). For the second (noradrenaline) peak, the average time from electrical stimulus to the peak for the two protocols was 636 ± 6 ms ($n = 7$), 627 ± 5 ms ($n = 7$) and 649 ± 5 ms ($n = 7$), corresponding to 0, 1.2 and 3 mM Mg^{2+}, respectively.

The rising and falling Mg^{2+} concentration protocol data were combined to show the overall effect of Mg^{2+} concentration on change in force of contraction (Δ force; Fig. 3B). While the peak Δ force was similar for ATP and noradrenaline peaks in 0 mM Mg^{2+}, normal (1.2 mM) and high (3 mM) Mg^{2+} concentrations markedly attenuated the ATP peak, but only high Mg^{2+} concentrations decreased the noradrenaline peak (Fig. 3B).

To exploit the benefits of within tissue responses we normalised the force of contraction and time to peak contraction to 100% in each tissue in the presence of normal PSS (Mg^{2+} 1.2 mM). For the combined protocols, the time to peak contraction was generally unaltered by changing Mg^{2+} concentration except for the ATP peak where there was an increase in time of 107 ± 2% when the Mg^{2+} was lowered from 1.2 to 0 mM ($P = 0.03$) and for the noradrenaline contraction where there was a small but significant increase in time to peak of 104 ± 0.7% when the Mg^{2+} was raised from 1.2 to 3 mM ($P = 0.005$; Fig. 3C).

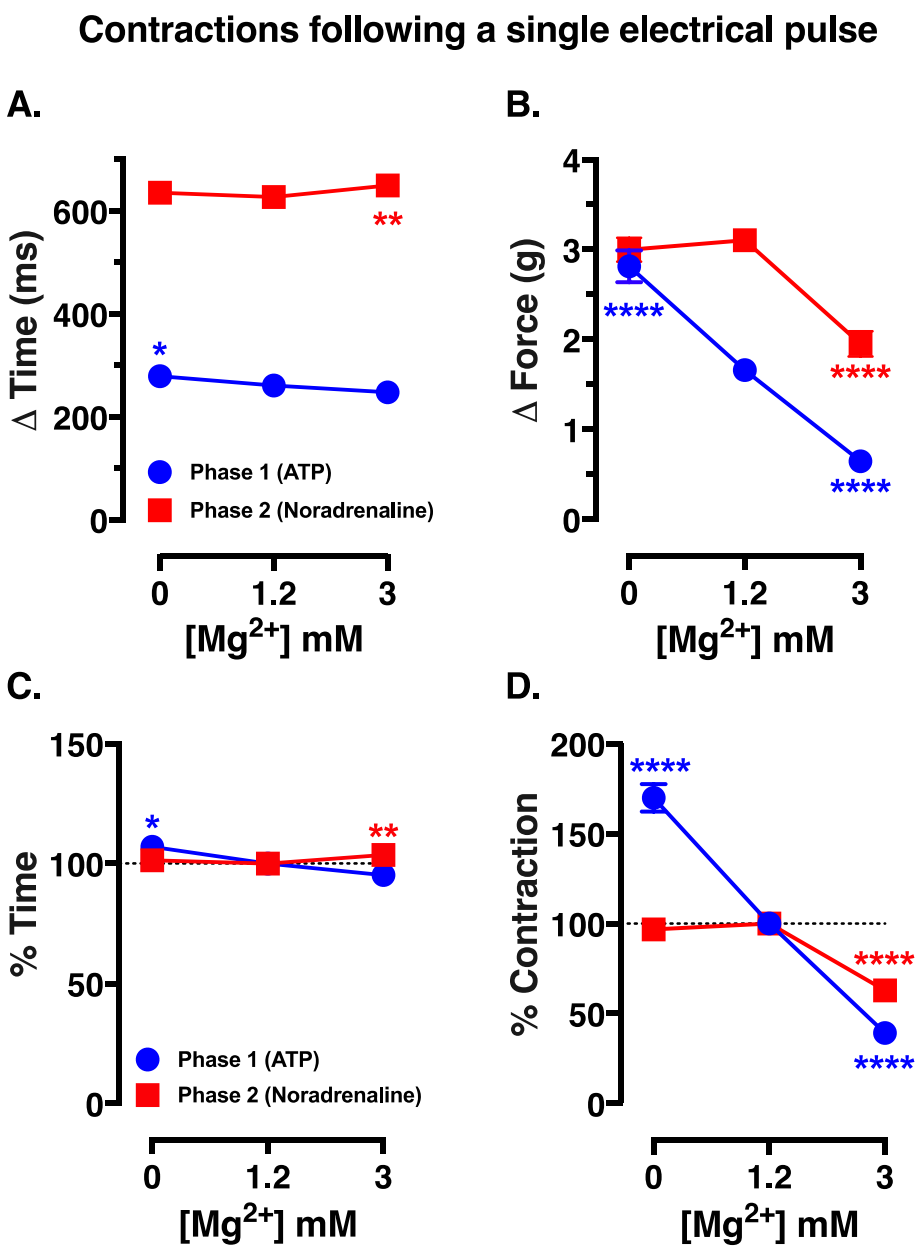

Fig. 3. Contractions of rat isolated vasa deferentia following a single electrical field pulse in the presence of low, normal or high Mg^{2+} concentrations. Responses shown are the combined data from the rising and falling Mg^{2+} concentration protocols of 0, 1.2 and 3 mM. A. Time from electrical stimulus to the peak contraction (Δ time, ms) for the phase 1 ATP-mediated contraction ($n = 6$) or the phase 2 noradrenaline-mediated contraction ($n = 7$). B. Peak increase in force (Δ force, g) for the phase 1 ATP-mediated contraction ($n = 10$) or the phase 2 noradrenaline-mediated contraction ($n = 10$). C. Time from electrical stimulus to the peak contraction expressed as a percentage of the respective responses taken as 100% in PSS containing a normal Mg^{2+} concentration (1.2 mM) for the phase 1 ATP-mediated contraction ($n = 6$) or the phase 2 noradrenaline-mediated contraction ($n = 7$). D. Peak increase in force expressed as a percentage of the respective responses taken as 100% in physiological salt solution (PSS) containing a normal Mg^{2+} concentration (1.2 mM) for the phase 1 ATP-mediated contraction ($n = 10$) or the phase 2 noradrenaline-mediated contraction ($n = 10$). Values are mean ± S.E.M. (error bars not shown are contained within the symbol). *n*, number of tissues from separate rats. Repeated measures one-way ANOVA with Dunnett *post hoc* test was performed; $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{****}P < 0.0001$ compared to respective Mg^{2+} 1.2 mM (normal) group.

The phase 1 ATP contraction was 170 ± 8% ($n = 10$) in 0 mM Mg^{2+} compared with normal Mg^{2+} (1.2 mM) and only 39 ± 3% ($n = 10$) in high Mg^{2+} (3 mM; Fig. 3D). In contrast, the noradrenaline peak contraction (phase 2) was not affected by 0 mM Mg^{2+} at 97 ± 3% ($n = 10$) of responses in normal Mg^{2+}, but was decreased significantly in high Mg^{2+} 3 mM to 63 ± 4% ($n = 10$); however, this was only half the attenuation observed in the ATP contraction (Fig. 3D).

3.3. Effects of Mg^{2+} concentration on the prejunctional sensitivity to ω-conotoxin GVIA

ω-Conotoxin GVIA is a potent and highly selective, concentration-dependent inhibitor of the prejunctional N-type ($Ca_v2.2$) calcium channel. In Mg^{2+} 0 mM and in the presence of prazosin 100 nM, the ATP-mediated contraction to a single field pulse was decreased to zero by increasing concentrations of ω-conotoxin GVIA (10^{-10} to 10^{-7} M; Fig. 4A). The pIC_{50} was 8.13 ± 0.04. As the Mg^{2+} concentration was elevated to 1.2 or 3 mM, the contractions in the absence of ω-conotoxin GVIA (baseline) were decreased as observed before (see Fig. 3B) and the ω-conotoxin GVIA IC_{50} shifted left. The small left shift in ω-conotoxin GVIA IC_{50} with 0–3 mM Mg^{2+} was 1.7-fold ($P = 0.018$; Fig. 4A).

In the presence of NF449 (10 μM), the noradrenaline-mediated control contraction to a single electrical pulse was unaffected by the rise in Mg^{2+} from 0 to 1.2 mM, but significantly decreased in 3 mM Mg^{2+} (baseline, Fig. 4B), as observed in Fig. 3B. The pIC_{50} for ω-conotoxin GVIA (8.26 ± 0.06 in Mg^{2+} 0 mM) was slightly left-shifted as the Mg^{2+} concentration increased to 3 mM, but the shift of 1.4-fold was not significant ($P = 0.28$; Fig. 4B).

3.4. Effects of Mg^{2+} concentration on exogenous agonist-induced contractions

To study the effects of Mg^{2+} concentration on the contraction response to exogenous ATP (100 μM) and separately noradrenaline (10 μM) or methoxamine (10 μM), the rising concentration (Mg^{2+} 0, 1.2 and 3 mM) and falling concentration (Mg^{2+} 3, 1.2 and 0 mM) protocols were followed. It was clearly evident that ATP 100 μM caused a small, transient peak contraction in normal Mg^{2+} (1.2 mM) PSS of

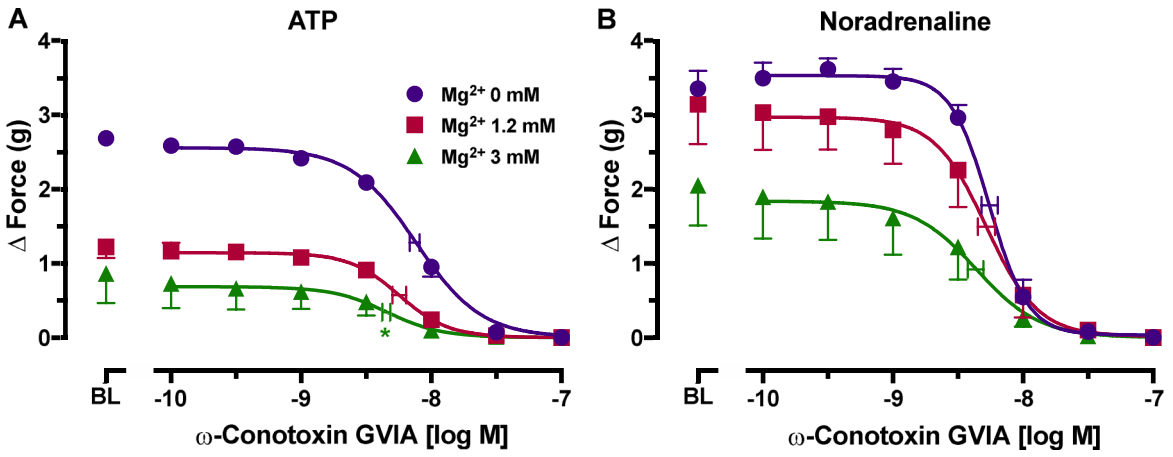

Fig. 4. Contractions of rat isolated vasa deferentia following a single electrical field pulse in the presence of low (0 mM), normal (1.2 mM) and high (3 mM) Mg^{2+} and increasing concentrations of the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA. Peak increases in force (Δ force in g; mean ± SEM, $n = 3$) are shown (A) for the phase 1 ATP-mediated contraction in the presence of prazosin 100 nM and (B) for the phase 2 noradrenaline-mediated contraction in the presence of NF449 10 μM. Every 30 min the single pulse was repeated in the presence of ω-conotoxin GVIA (10^{-10} to 10^{-7} M). BL, baseline contraction to a single electrical field pulse, in each respective Mg^{2+} concentration, before addition of ω-conotoxin GVIA. Horizontal bars are ± 1 S.E.M. of the individual fitted IC_{50} values placed at the mean for each line. The left shift of the ω-conotoxin GVIA IC_{50} on the ATP-mediated contraction for 0 to 3 mM Mg^{2+} was significant (*$P = 0.018$).

0.22 ± 0.03 g ($n = 15$) compared with the peak contraction to a single field pulse of 1.66 ± 0.09 g ($n = 10$). This very weak response to exogenous ATP compared with a single electrical field pulse contrasts starkly with the contraction to 10 μM noradrenaline. Here the peak contraction was 1.1 ± 0.08 g ($n = 16$) in normal Mg^{2+} 1.2 mM compared with the contraction response to a single electrical pulse of 3.1 ± 0.1 g ($n = 10$).

Evidently the contraction to the high exogenous ATP concentration (100 μM) could not come close (only 13%) to mimicking the contraction to the ATP released from the single pulse (in normal Mg^{2+} PSS). For exogenous noradrenaline or methoxamine, the maximum contraction was 35% and 40%, respectively, of that induced by the single electrical field pulse (in normal Mg^{2+} PSS). Noting these important differences in the scale of contraction to ATP and noradrenaline from the nerve-released transmitters compared with the contraction to exogenous ATP, noradrenaline and methoxamine, we again observed that exogenous ATP was far more affected by Mg^{2+} than exogenous noradrenaline or methoxamine especially moving from normal 1.2 mM to 0 mM Mg^{2+} or from normal to high Mg^{2+} (3 mM; Fig. 5).

For the combined protocols in the Mg^{2+}-free PSS, the ATP contraction was 147 ± 11% ($n = 15$; $P = 0.002$) of the contraction in normal Mg^{2+} PSS and was significantly less in 3 mM Mg^{2+} (55 ± 6%, $n = 15$; $P < 0.0001$; Fig. 5). Noradrenaline was again not significantly affected by Mg^{2+} 0 mM (118 ± 8%, $n = 16$; $P = 0.08$) compared with normal (1.2 mM) Mg^{2+} but was significantly attenuated in 3 mM Mg^{2+} to 83 ± 3% ($n = 16$; $P = 0.0005$; Fig. 5). Similarly, the contraction to methoxamine was not significantly affected by Mg^{2+} 0 mM (112 ± 10%, $n = 6$, $P = 0.56$) compared with normal (1.2 mM) Mg^{2+} and tended to be attenuated in 3 mM Mg^{2+} to 74 ± 12% ($P = 0.11$; Fig. 5).

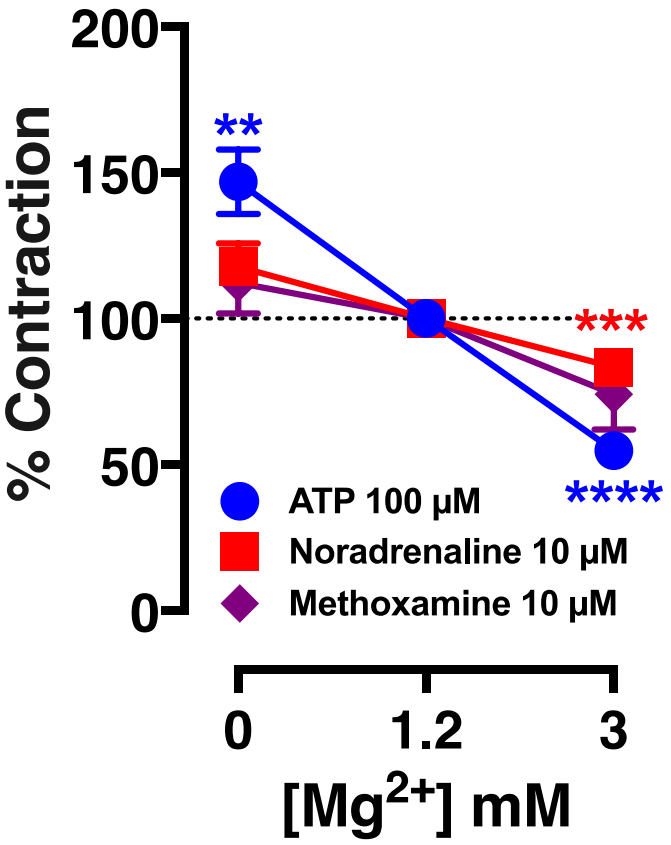

Fig. 5. Contractions of rat isolated vasa deferentia following administration of exogenous ATP, noradrenaline or methoxamine in the presence of low, normal or high Mg^{2+} concentrations; data from the rising and falling Mg^{2+} concentration protocols of 0, 1.2 and 3 mM have been combined. Responses are peak increases in force expressed as a percentage of the respective contraction taken as 100% in PSS containing a normal Mg^{2+} concentration (1.2 mM) for ATP 100 μM ($n = 15$), noradrenaline 10 μM ($n = 16$) or methoxamine 10 μM ($n = 6$). Values are mean ± S.E.M. (error bars not shown are contained within the symbol). n, number of tissues from separate rats. Repeated measures one-way ANOVA with Dunnett *post hoc* test was performed; **$P < 0.01$, ***$P < 0.001$ or ****$P < 0.0001$ compared to respective Mg^{2+} 1.2 mM (normal) group.

4. Discussion

We found that lowering the Mg^{2+} concentration from 1.2 mM to 0 mM markedly enhanced the first peak of contraction of the rat isolated vas deferens but had no effect on the second peak of the contraction in response to a single electrical pulse. We have confirmed that the first peak of the contraction is solely due to ATP as it was abolished by the P2X1 receptor antagonist NF449 and that the second peak was entirely due to noradrenaline acting at prazosin-sensitive α_1-adrenoceptors.

The concentration of NF449, 10 μM, was shown to be selective for

ATP P2X1 receptors (pK_B 6.4) with no activity at α_1-adrenoceptors (Angus and Wright, 2015). Prazosin, a competitive α_1-adrenoceptor antagonist, inhibits nerve-mediated or phenylephrine-mediated contractions in rat or mouse small resistance arteries with a pK_B 9.1 (Angus and Wright, 2015).

The time from the application of the electrical stimulus to the first and second peak gives an indication of the relative time to activation of intracellular Ca^{2+} through the ionotropic P2X1 receptor for ATP at 260 ms compared with the slower metabotropic α_1-adrenoceptor coupled through inositol triphosphate (Purves et al., 2001) at 627 ms for noradrenaline. Our results show that changing the Mg^{2+} concentration made some small but significant increases or decreases in the time to peak contraction. However, the underlying result suggests that the peak ATP-mediated contraction occurs some 2.4 times faster than the noradrenaline peak. Noting the difference in the ATP ionotropic P2X1 receptor and the noradrenaline G-protein-coupled metabotropic α_1-adrenoceptor, it is not surprising that lowering Mg^{2+} to zero would remove the competition between Mg^{2+} and Ca^{2+} at the ionotropic P2X1 receptor allowing more Ca^{2+} to flow and enhance the contraction. At the high Mg^{2+} 3 mM concentration, Ca^{2+} would be less available through the P2X1 receptor and to a lesser degree in association with the α_1-adrenoceptor activation mechanism.

4.1. Exogenous ATP, noradrenaline and methoxamine

We tested a high concentration of ATP (100 μM), noradrenaline (10 μM) and methoxamine (10 μM) as a comparison with the twin peak contractions to a single nerve stimulation. However, it is clear that bath-applied agonists cannot mimic the transient high local concentration of ATP and noradrenaline released in a tight synaptic cleft estimated at 20 nm (Burnstock, 1990). ATP 100 μM caused a weak contraction compared with the first phase contraction in response to the single field pulse. Equally, noradrenaline 10 μM was a poor agonist compared to the phase 2 contraction. These poor contractions will be the resultant of slow diffusion through a thick tissue, metabolism and possibly desensitisation. Nevertheless, the exogenous agonist contractions were similarly affected by the presence of 0, 1.2 or 3 mM Mg^{2+} (Fig. 5).

In pilot experiments we tested the more stable αβ-methylene ATP in an attempt to mimic the contraction to ATP. However, the rapid desensitisation of the P2X1 receptors and lack of subsequent responses to the ligand caused this approach to be abandoned. Methoxamine on the other hand is a useful tool as it is a selective α_1-adrenoceptor agonist devoid of β-adrenoceptor and α_2-adrenoceptor activity; it is not subject to neuronal uptake and metabolism that could have affected the contractions to exogenous noradrenaline. We show here that the contraction to methoxamine was affected by high Mg^{2+} similarly to that for exogenous noradrenaline.

4.2. Cotransmission

With modern technology we can separate the cotransmitter-dependent contractions following a single electrical pulse into the 2 components of ATP and noradrenaline. This work cannot shed any light on the 3 hypotheses that 1) ATP and noradrenaline are co-stored in the one vesicle; 2) ATP and noradrenaline are stored in separate vesicles; or 3) that they are located in separate varicosities (Burnstock, 1990).

4.3. Sites of action of Mg^{2+}

The contraction to a single field pulse could be affected by Mg^{2+} acting at the release site on the sympathetic varicosity or on the post-junctional receptor and its smooth muscle effector machinery, or both sites. We have used two approaches to shed some light as to the mechanism of the effect of Mg^{2+} on the single pulse contraction. First, ω-conotoxin GVIA is a highly selective N-type $Ca_v2.2$ calcium channel antagonist. In rat mesenteric small resistance arteries ω-conotoxin GVIA 3 nM completely inhibited excitatory junction potentials and contractions to trains of perivascular nerve stimulation with no effect on the concentration-response curve to KCl (10–70 mM) or noradrenaline (0.1–30 μM) (Pruneau and Angus, 1990). Even as high as 10 μM, ω-conotoxin GVIA had no effect on contractions to exogenous noradrenaline (0.1–30 μM) in rat small mesenteric arteries (Whorlow et al., 1996). Thus, the sole site of action of ω-conotoxin GVIA would be to inhibit the release of neural transmitters. We show here that the IC_{50} for ω-conotoxin GVIA was similar for the ATP- and noradrenaline-mediated contractions in 0 mM Mg^{2+} and that raising Mg^{2+} to 3 mM caused only a small increase in sensitivity of < 2-fold, which was just significant for ATP. Importantly, there was no difference in the ω-conotoxin GVIA IC_{50} between 0 and 1.2 mM Mg^{2+} where there is the large fall in the contraction to ATP. The results of this experiment would suggest that the main sites of action of Mg^{2+} are at the post-junctional effector receptors for ATP and noradrenaline. Given the limitations of trying to mimic neural-released ATP and noradrenaline with exogenous ATP, noradrenaline and methoxamine, our study strongly suggests that Mg^{2+} directly competes with Ca^{2+} involved in the ATP P2X1 receptor-mediated contraction, but only at the high (3 mM) concentration does it inhibit the α_1-adrenoceptor-mediated contraction.

4.4. Conclusion

Our work clearly demonstrates that the contractions to each of the cotransmitters ATP and noradrenaline in the rat vas deferens can be determined and are differently altered by low Mg^{2+} concentration, but both are inhibited by high Mg^{2+} concentration. The sites of action of Mg^{2+} are most probably at the post-junctional P2X1 receptor and the α_1-adrenoceptor. The ATP-mediated contraction is more sensitive to changing Mg^{2+} concentrations than the noradrenaline-mediated contraction consistent with the P2X1 receptor coupled to an ionotropic channel. Our results suggest that this assay with a single electrical field pulse is ideal to test various pre- or post-junctional modulators of cotransmitter release. We suggest that any future publications should carefully justify why the magnesium concentration in PSS should be other than normal 1.2 mM.

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Declaration of competing interest

None.

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