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RESEARCH PAPER

$P2Y_1$ receptors mediate inhibitory neuromuscular transmission in the rat colon

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Background and purpose: Inhibitory junction potentials (IJP) are responsible for smooth muscle relaxation in the gastrointestinal tract. The aim of this study was to pharmacologically characterize the neurotransmitters [nitric oxide (NO) and adenosine triphosphate (ATP)] and receptors involved at the inhibitory neuromuscular junctions in the rat colon using newly available $P2Y_1$ antagonists.
Experimental approach: Organ bath and microelectrode recordings were used to evaluate the effect of drugs on spontaneous mechanical activity and resting membrane potential. IJP and mechanical relaxation were studied using electrical field stimulation (EFS).
Key results: N^{ω}-nitro-L-arginine (L-NNA) inhibited the slow component of the IJP and partially inhibited the mechanical relaxation induced by EFS. MRS2179, MRS2500 and MRS2279, all selective $P2Y_1$ receptor antagonists, inhibited the fast component of the IJP without having a major effect on the relaxation induced by EFS. The combination of both L-NNA and $P2Y_1$ antagonists inhibited the fast and the slow components of the IJP and completely blocked the mechanical relaxation induced by EFS. Sodium nitroprusside caused smooth muscle hyperpolarization and cessation of spontaneous motility that was prevented by oxadiazolo[4,3-α]quinoxalin-1-one. Adenosine 5′-O-2-thiodiphosphate, a preferential P2Y agonist, hyperpolarized smooth muscle cells and decreased spontaneous motility. This effect was inhibited by $P2Y_1$ antagonists.
Conclusions and implications: The co-transmission process in the rat colon involves ATP and NO. $P2Y_1$ receptors mediate the fast IJP and NO the slow IJP. The rank order of potency of the $P2Y_1$ receptor antagonists is MRS2500 greater than MRS2279 greater than MRS2179. $P2Y_1$ receptors might be potential pharmacological targets for the regulation of gastrointestinal motility.
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Keywords: smooth muscle; gastrointestinal; inhibitory neuromuscular transmission; $P2Y_1$ receptors; nitric oxide; MRS2179; MRS2279; MRS2500

Abbreviations: ADPβS, adenosine 5′-O-2-thiodiphosphate; AUC, area under the curve; EFS, electrical field stimulation; GI, gastrointestinal; IJP, inhibitory junction potential; IJPf, fast component of the IJP; IJPs, slow component of the IJP; L-NNA, N^{ω}-nitro-L-arginine; ODQ, oxadiazolo[4,3-α]quinoxalin-1-one; SNP, sodium nitroprusside

Introduction

Stimulation of inhibitory motor neurones by electrical field stimulation (EFS) causes the release of inhibitory neurotransmitter(s). Smooth muscle responds with a transient inhibitory junction potential (IJP), which is the electrophysiological base of smooth muscle relaxation. IJP usually display two components: a fast (IJPf) followed by a slow (IJPs) component. These two components suggest the release of at least two inhibitory neurotransmitters (Crist *et al.*, 1992; Lyster *et al.*, 1992; He and Goyal, 1993; Keef *et al.*, 1993; Pluja *et al.*, 1999; Gallego *et al.*, 2006). Experiments with new pharmacological tools are essential to characterize the co-transmission process where nitric oxide (NO) and ATP might be involved (Burnstock, 2008; Gallego *et al.*, 2008a). It has been clearly demonstrated that the IJPs is abolished by treatments that block NO synthesis (Keef *et al.*, 1993; Pluja *et al.*, 1999; Wang *et al.*, 2007); this indicates that the IJPs is mediated by NO release from inhibitory motor neurones. In contrast, NO synthase inhibition does not alter the IJPf, showing that this component is not mediated by NO.

ATP, or a related purine, is an inhibitory neurotransmitter in the gut (Burnstock *et al.*, 1970; Ralevic and Burnstock, 1998). There are two families of purine receptors: P2X and P2Y. P2X receptors are ligand-gated ion channels, and P2Y

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receptors are G-protein coupled receptors. Eight distinct P2Y receptor subtypes, $P2Y_{1,2,4,6,11,12,13,14}$ and seven P2X receptor subtypes, $P2X_{1-7}$, have been reported (Burnstock, 2007).

MRS2179 is a selective antagonist of $P2Y_1$ receptors that has been recently used to characterize purinergic inhibitory neurotransmission in the gastrointestinal (GI) tract (Boyer *et al.*, 1998; Camaioni *et al.*, 1998; Alexander *et al.*, 2008). However, findings are not conclusive, and the effect of MRS2179 might vary between species and areas of the GI tract. In the human colon (Gallego *et al.*, 2006; 2008a), guinea pig ileum (Wang *et al.*, 2007) and pig small intestine (Gallego *et al.*, 2008b), the IJPf is sensitive to MRS2179. In rodents, the non-nitrergic relaxation is partially sensitive to MRS2179; the response of circular muscle strips of mouse jejunum are affected by MRS2179 at a concentration of 1 μM (De Man *et al.*, 2003). Moreover, adenosine 5′-O-2-thiodiphosphate (ADPßS), a preferential P2Y agonist, induces MRS2179-sensitive relaxations in the rat distal colon. It has thus been postulated that post-junctional $P2Y_1$ receptors located in smooth muscle mediate the non-nitrergic relaxation in murine tissues (Giaroni *et al.*, 2002; Van Crombruggen *et al.*, 2007). Higher concentrations of MRS2179 (10 μM) are needed to inhibit the IJPf in the mouse internal anal sphincter (McDonnell *et al.*, 2008) and caecum (Zizzo *et al.*, 2007). These results suggest that other P2Y receptors might be involved in inhibitory neurotransmission (McDonnell *et al.*, 2008), or, alternatively, pre-junctional $P2Y_1$ receptors might mediate this effect (Zizzo *et al.*, 2007).

Specific P2Y receptor antagonists are essential pharmacological tools for the proper characterization of purinergic inhibitory neurotransmission (Bornstein, 2008). In this study, we investigated inhibitory neurotransmission in the rat mid-colon using MRS2179 (Camaioni *et al.*, 1998), and, for the first time, two other recently available $P2Y_1$ antagonists, MRS2279 (Boyer *et al.*, 2002) and MRS2500 (Kim *et al.*, 2001); (Cattaneo *et al.*, 2004). Briefly, we found that the IJPf and the non-nitrergic relaxation evoked by EFS were inhibited in a concentration-dependent manner by MRS2179, MRS2500 and MRS2279, indicating the involvement of $P2Y_1$ receptors in the purinergic inhibitory neurotransmission in the rat mid-colon. Further study of these $P2Y_1$ receptors is crucial to establish them as pharmacological targets in the regulation of gastrointestinal functions, such as secretion and motility (Wood, 2006).

Methods

Animals

Male Sprague-Dawley rats (300–350 g), 8–10 weeks old, were kept at a constant temperature (19–21°C) and humidity (60%), with a lighting cycle of 12 h light/12 h dark and had access to water and food *ad libitum*. The rats were killed by stunning, a sharp blow to the head, before being decapitated and bled. This procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Tissue preparation

The mid-colon was quickly removed and placed in carbogenated Krebs solution. The mesenteric fat was removed, and the colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing upward. The mid-colon was distinguished according to the longitudinal orientation of the folds of the mucosa (total length about 5 cm in the centre of the colon) according to anatomical criteria previously described (Alberti *et al.*, 2005). Mucosal and submucosal layers were carefully removed, and the circular muscle was cut into strips 1 cm long and 0.3 cm wide.

Intracellular microelectrode recording

Muscle strips were pinned to the base of a Sylgard-coated chamber, circular muscle side up, and continuously perfused with Krebs solution. Strips were allowed to equilibrate for approximately 1 h before the recording commenced. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl (30–60 MΩ of resistance). Membrane potential was measured using standard electrometer Duo773 (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) using PowerLab 4/30 system and Chart 5 software for Windows (all from ADInstruments, Castle Hill, NSW, Australia). EFS was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. The EFS had the following parameters: total duration of train, 100 ms; frequency, 20 Hz; pulse duration, 0.3 ms, and increasing amplitude voltage, 5, 10, 12, 15, 17, 20, 25, 30 and 50 V. The amplitude and the duration of the EFS-induced IJP were measured under control conditions and after infusion of each drug. Resting membrane potential was also measured before and after drug addition. Nifedipine (1 μM) was used to abolish the mechanical activity and obtain stable impalements.

Mechanical studies

Spontaneous mechanical activity was studied in a 10 mL organ bath. Circularly orientated preparations were tied to a support at one end and to an isometric force transducer (Harvard VF-1, Harvard Apparatus Inc., Holliston, MA, USA) at the other using a 2/0 silk thread. Mechanical activity was recorded by means of the transducer, which was connected to a personal computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter. A tension of 1 g was applied, and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. In order to study the inhibitory neurotransmitters released, EFS was applied for 4 min (pulse duration, 0.3 ms; frequency, 5 Hz; amplitude, 30 V) through two platinum electrodes placed on the support holding the tissue. To estimate mechanical activity responses to drugs or EFS, the area under the curve (AUC) of contractions from the baseline was measured before and after drug addition and before and during EFS. AUC is expressed as $g \cdot min^{-1}$.

Solutions and drugs

The composition of the Krebs solution was (in mM): glucose, 10.10; NaCl, 115.48; $NaHCO_3$, 21.90; KCl, 4.61; NaH_2PO_4,

1.14; $CaCl_2$, 2.50 and $MgSO_4$, 1.16 (pH 7.3–7.4). The Krebs solution (37 ± 1°C) was bubbled with carbogen (95% O_2 and 5% CO_2). In all the experiments, phentolamine, atropine and propanolol 1 μM was added to the Krebs solution to block α- and β-adrenoceptors and muscarinic receptors.

The following drugs were used: nifedipine, N^{ω}-nitro-L-arginine (L-NNA), ADPβS, phentolamine, sodium nitroprusside (SNP) 1H-[1,2,4], oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (Sigma Chemicals, St. Louis, MO, USA), atropine sulphate (Merck, Darmstadt, Germany), propanolol, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS2179), (1R,2S,4S,5S)-4-[2-chloro-6-(methylamino)-9H-purin - 9 - yl] - 2 - (phosphonooxy)bicyclo [3.1.0] hexane - 1-methanol dihydrogen phosphate ester diammonium salt (MRS2279), and (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H - purin - 9 - yl] - 2 - (phosphonooxy)bicyclo [3.1.0] hexane - 1-methanol dihydrogen phosphate ester diammonium salt (MRS2500) (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water, except for nifedipine and ODQ, which were dissolved in 96% ethanol and L-NNA, which was dissolved in Krebs solution by sonication. Drug and receptor nomenclature conform to the guidelines of the *British Journal of Pharmacology* (Alexander *et al*., 2008)

Data analysis and statistics

Differences in the resting membrane potential before and after infusion of different drugs were compared by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. The duration of the IJP was measured from the beginning of the hyperpolarization to the value of a stable resting membrane potential. The differences between the amplitude and duration of the IJPs before and after drug infusion were compared by two-way ANOVA (drug and voltage).

To normalize mechanical data, the effect of drugs and EFS were calculated as percentage of inhibition, being 100% when a total inhibition of spontaneous motility was recorded after drug administration or during EFS, and 0% when the inhibitory response was completely antagonized. Rebound contraction recorded at the end of the timulus was normalized with the average amplitude of spontaneous contractions before EFS. One-way ANOVA was used (i) to evaluate the effect of drugs on inhibition of spontaneous motility induced by SNP or ADPßS; and (ii) to evaluate the effect of different antagonists on inhibition of spontaneous motility and rebound contraction evoked by EFS. Paired *t*-test was used to compare data when two single groups were studied.

Data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant; *n* values indicate the number of samples. Statistical analysis was performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

Results

Role of nitric oxide synthase inhibitors in the IJP and relaxation induced by EFS

EFS completely inhibited spontaneous motility, and a rebound contraction (off-contraction) was recorded after the end of the stimulus. The amplitude of the off-contraction was 1.51 ± 0.11 (n = 18) higher than the mean spontaneous contraction. L-NNA (1 mM, n = 11) partially decreased the inhibitory effect induced by EFS by about 23 ± 6% (Figure 1A). However, L-NNA (1 mM, n = 11) did not significantly modify the rebound contraction [control: 1.66 ± 0.14 vs. L-NNA: 1.60 ± 0.07; *t*-test not significant (n.s.)]. As previously reported, EFS caused an IJP with two components: an IJPf (measured as the IJP amplitude) followed by an IJPs (measured as the IJP duration at baseline) (Pluja *et al.*, 1999; control tracings of Figures 2 and 4). Both the amplitude and duration of the IJP were voltage dependent. Neither L-NNA (1 mM, n = 4) nor the guanylate cyclase inhibitor ODQ (10 μM, n = 8) modified the amplitude of the IJP, but they did reduce its duration (ANOVA $P < 0.0001$), that is 50 V: control: 3.4 ± 0.4 s versus L-NNA: 2.3 ± 0.2 s and control: 3.7 ± 0.3 s versus ODQ: 2.7 ± 0.3 s. The remaining IJP was the IJPf that is sensitive to $P2Y_1$ antagonists (see below).

Role of $P2Y_1$ antagonists in the IJP and relaxation induced by EFS

Muscle bath experiments demonstrated that MRS2179 (10 μM, n = 7 and 20 μM, n = 5), MRS2279 (1 μM, n = 5) and MRS2500 (1 μM, n = 5) did not modify the inhibitory effect induced by EFS (Figure 1B–D). The amplitude of the rebound contraction was not modified by the different $P2Y_1$ antagonists tested (paired *t*-test): that is control: 1.27 ± 0.17 versus MRS2179 (10 μM) 1.40 ± 0.25, (n = 7, n.s), control: 1.47 ± 0.30 versus MRS2179 (20 μM) 1.48 ± 0.32, (n = 5, n.s), control: 1.28 ± 0.20 versus MRS2279 (1 μM) 0.83 ± 0.23, (n = 5, n.s), and control: 1.35 ± 0.09 versus MRS2500 (1 μM) 0.88 ± 0.16, (n = 5, n.s.). The amplitude of rebound contraction was normalized in relation to spontaneous motility (see *Methods*). The electrophysiological experiments demonstrated that MRS2179 (20 μM; n = 6), MRS2279 (1 μM; n = 4) and MRS2500 (1 μM; n = 8) did not modify the duration but did markedly reduce the amplitude of the IJP (Figure 2). It is important to note that the inhibitory effect was more prominent in experiments performed with MRS2279 (1 μM) and MRS2500 (1 μM) than in those performed with MRS2179 (20 μM). In experiments with MRS2179, the IJPf was recovered after 20 min washout, but in experiments with MRS2279 and MRS2500, the IJPf was only partially recovered after washout.

Role of NOS inhibitors and $P2Y_1$ antagonists in the IJP and relaxation induced by EFS

In order to characterize the neurotransmission process, preparations were first incubated with L-NNA (1 mM), and then the effects of the $P2Y_1$ antagonists were studied. Muscle bath experiments show a concentration-dependent inhibition of EFS-induced relaxation (Figure 3). IC_{50} values were: MRS2179, 3.5 μM; MRS2279, 43.9 nM; MRS2500, 16.5 nM. Note, in the presence of high concentrations of the $P2Y_1$ antagonists, an initial contraction is seen at the onset of the stimulus, contrasting with the relaxation seen in control (Figure 3). In the presence of L-NNA, the rebound contraction was reduced in a concentration-dependent manner by the $P2Y_1$ receptor antagonists. MRS2279 and MRS2500 caused a total inhibition of rebound contraction at 1 μM (Bonferroni's multiple comparison test, control vs. 1 μM, $P < 0.001$ both). In the presence

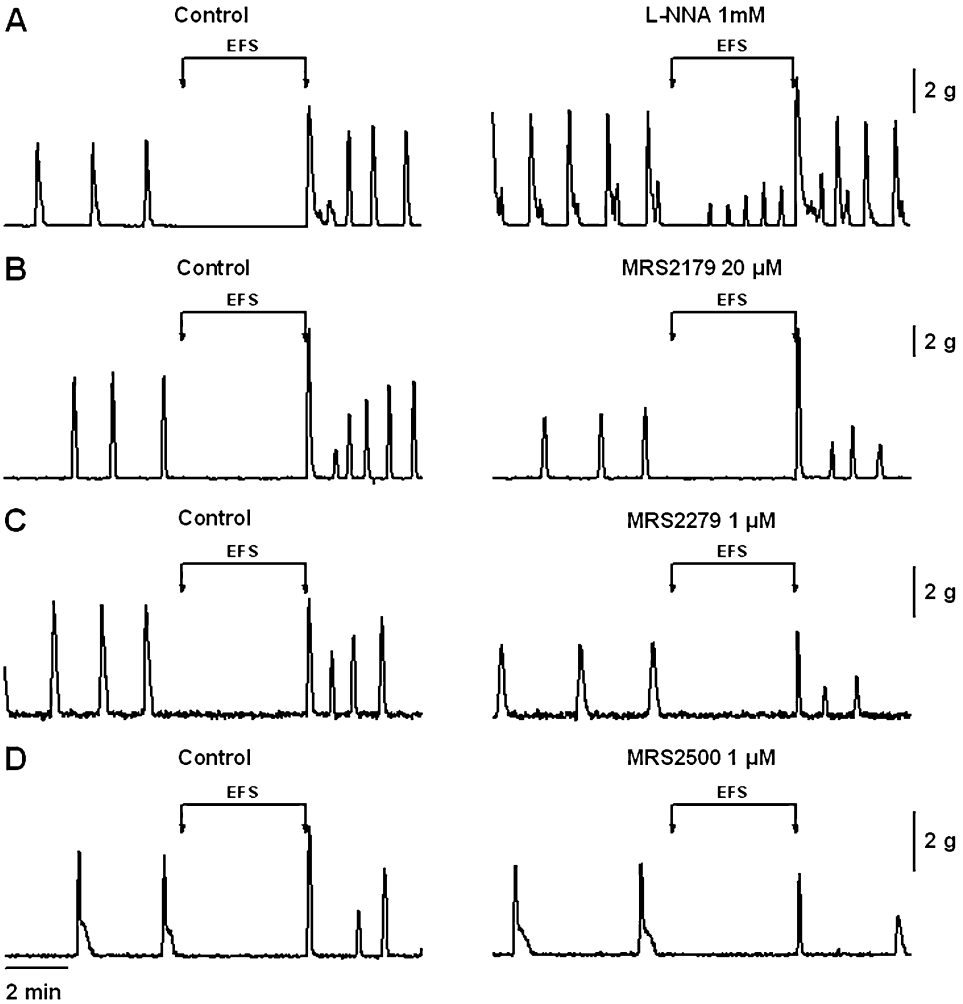

Figure 1 Muscle bath recordings showing the effect of N$^{\omega}$-nitro-L-arginine (L-NNA) 1 mM (A), MRS2179 20 μM (B), MRS2279 1 μM (C) and MRS2500 1 μM (D) on the inhibition of contractile activity induced by electrical field stimulation (EFS).

of both L-NNA and the $P2Y_1$ antagonists, a reduction of both the amplitude and the duration of the IJPs was observed (Figure 4). Concentration-response curves were performed in the presence of L-NNA (Figure 5). IC_{50} values were: MRS2179, 13.1 μM; MRS2279, 17.8 nM; MRS2500, 14.0 nM. Both 1 μM MRS2279 and 1 μM MRS2500 completely blocked the IJP. In contrast, a residual IJP was still recorded with 20 μM MRS2179.

Effect of NO donors on membrane potential and spontaneous motility

Incubation of the tissue with SNP (1 μM), an NO donor, caused a smooth muscle hyperpolarization (–11 ± 0.7 mV; $n = 18$) and a long-lasting (more than 10 min) cessation of spontaneous motility (AUC 11.40 ± 1.94 vs. 0.07 ± 0.05 g·min^{-1}; $n = 4$ *t*-test $P < 0.01$) (Figure 6 A,B respectively). The hyperpolarization induced by SNP 1 μM was not modified by pre-incubation with either L-NNA, MRS2500 or MRS2279 (Figure 6A). ODQ (10 μM) partially antagonized the hyperpolarization induced by SNP (Figure 6A). The inhibition of spontaneous motility induced by SNP (1 μM) was also not modified by either L-NNA, MRS2179, MRS2279 or MRS2500 (Figure 6B). In the presence of these antagonists, SNP (1 μM) caused a total inhibition (100%) of spontaneous motility. SNP caused only a partial inhibition of spontaneous motility (8.4 ± 2.2%; $P < 0.001$) in the presence of ODQ (10 μM) (Figure 6B).

Effect of ADPβS on membrane potential and spontaneous motility

Incubation of the tissue with ADPβS (10 μM) caused a transient (5 min) inhibition of spontaneous motility (AUC: 9.01 ± 1.47 vs. 4.04 ± 0.76 g·min^{-1}; $n = 14$, *t*-test $P < 0.001$) (Figure 7). This inhibitory effect was reversed by MRS2179, MRS2279 and MRS2500, but not by ODQ or L-NNA (Figure 7B). ADPßS (10 μM) induced a hyperpolarization of 9.15 ± 0.71 mV ($n = 25$), which was antagonized by MRS2179, MRS2279 and MRS2500 but not modified by pre-incubation with either L-NNA or ODQ (Figure 7A)

Discussion

In the present study, we have demonstrated that in the rat colon, the fast component of the IJP is sensitive to $P2Y_1$

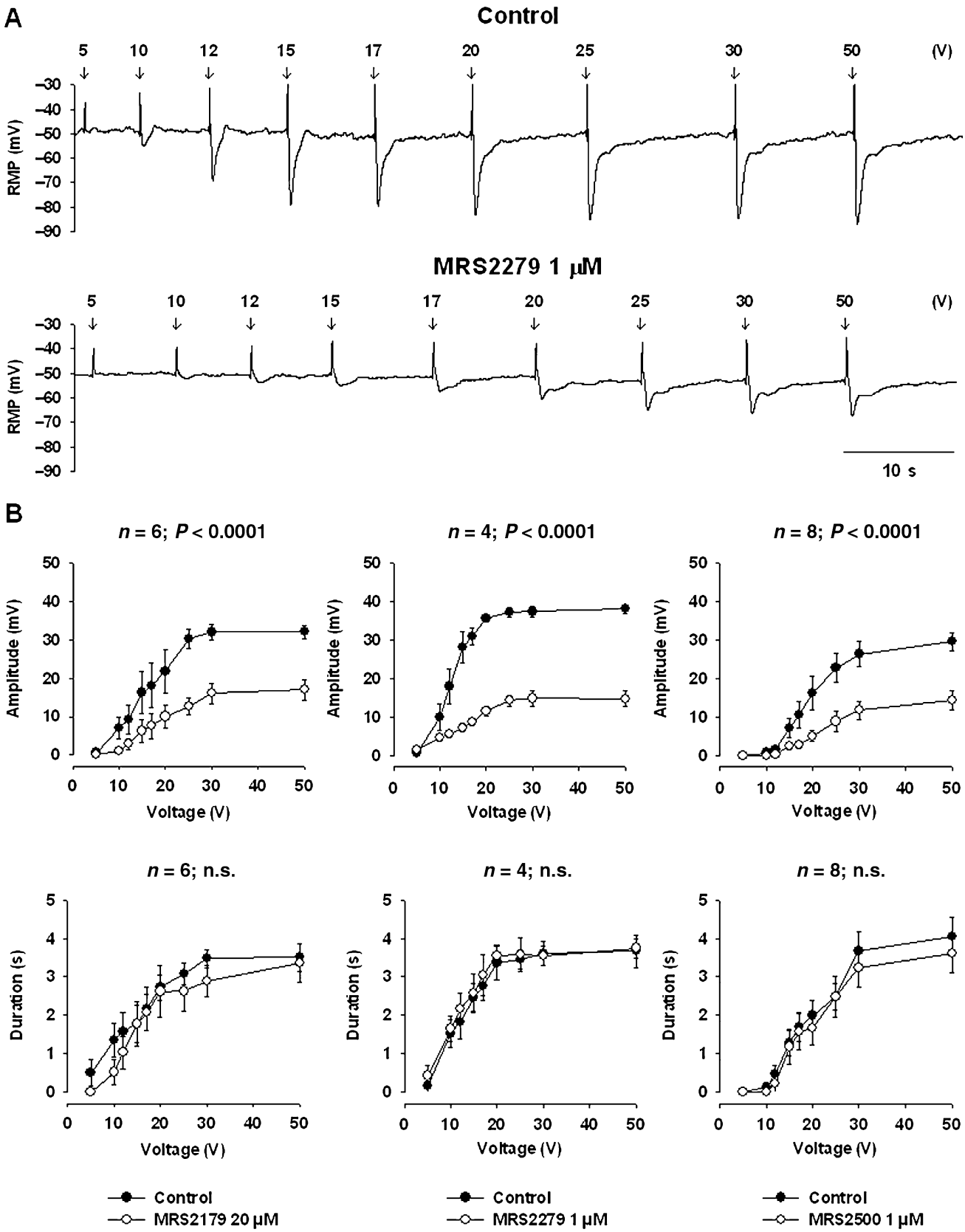

Figure 2 (A) Intracellular microelectrode recordings showing the electrical field stimulation (EFS)-induced inhibitory junction potential (IJP) at different voltages (5, 10, 12, 15, 17, 20, 25, 30 and 50 V) in control conditions and after incubation with MRS2279 (1 μM). (B) Graphs representing the inhibitory effect of MRS2179 (20 μM), MRS2279 (1 μM) and MRS2500 (1 μM) on the amplitude (top) and duration (bottom) of the EFS-induced IJP. All values are expressed as mean ± SEM. Significant differences were assessed using two-way ANOVA.

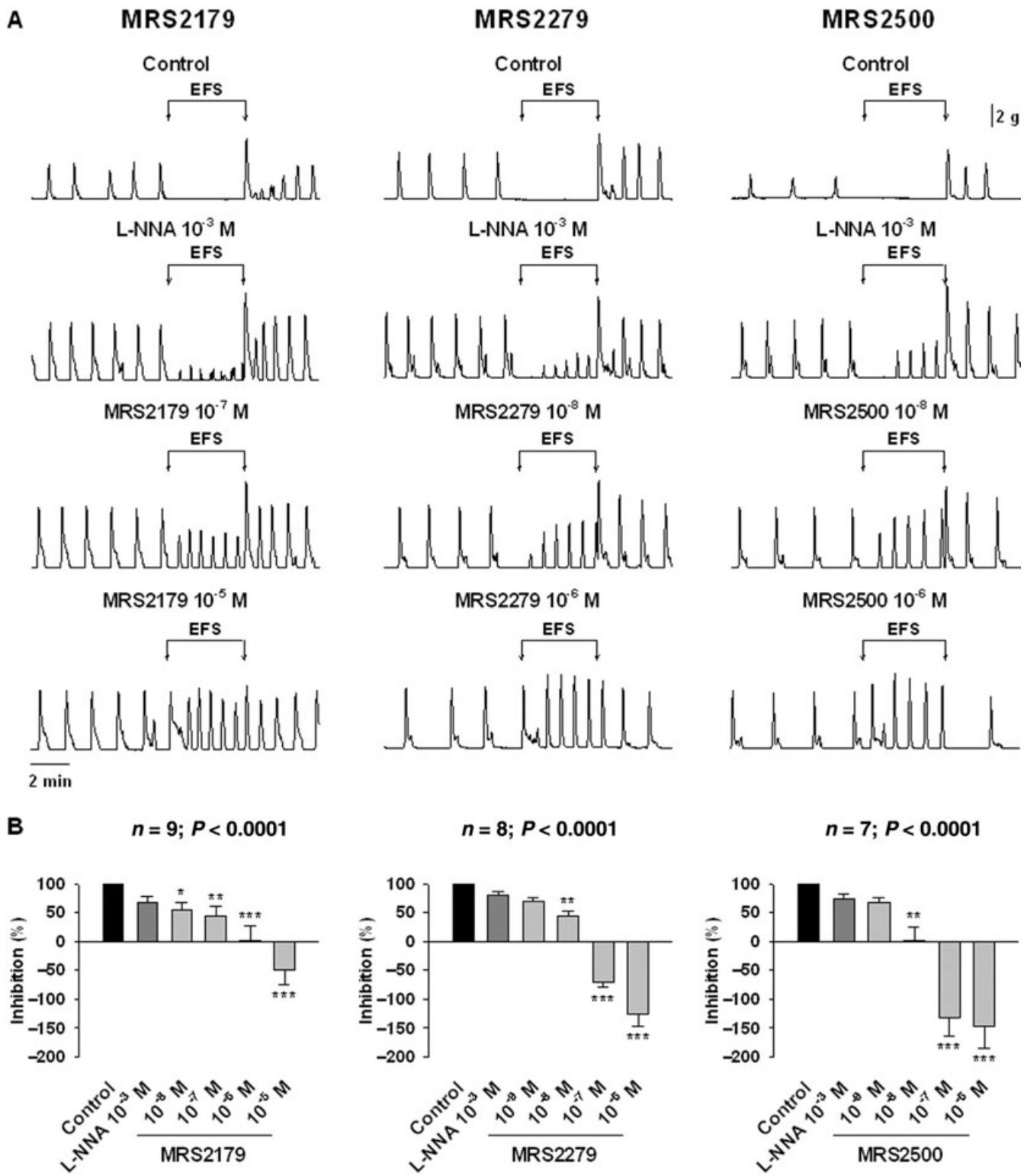

Figure 3 (A) Mechanical recordings showing the effect of MRS2179 (left), MRS2279 (middle) and MRS2500 (right) on the electrical field stimulation (EFS)-induced inhibition of spontaneous motility in the presence of N$^{\omega}$-nitro-L-arginine (1 mM) incubation. (B) Concentration-response histograms showing the percentage of inhibition. Data were calculated using the following formula: 1 – [area under the curve (AUC) during EFS/AUC previous EFS] × 100. Note that 100% is no drug effect (total inhibition of spontaneous motility) and 0% is a complete blockade of the inhibitory response, meaning that spontaneous motility during EFS is equal to spontaneous motility before EFS. Negative data indicate contractile activity during EFS, that is –100% represents a doubling of the spontaneous motility recorded prior to EFS. All values are mean ± SEM. MRS2179 $n = 9$, MRS2279 $n = 8$, MRS2500 $n = 7$. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significant difference from control.

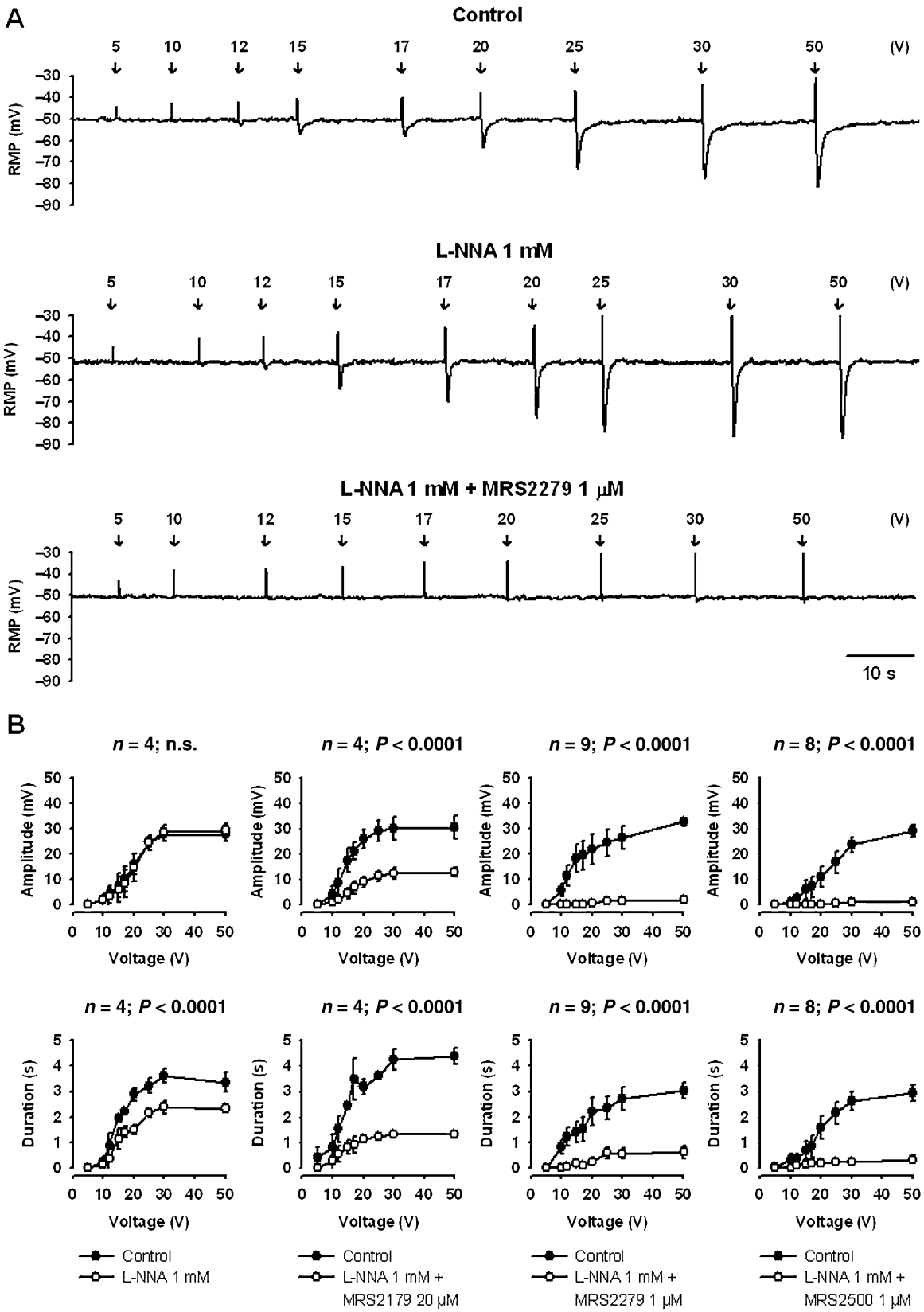

Figure 4 (A) Intracellular microelectrode recordings showing the electrical field stimulation (EFS)-induced inhibitory junction potential (IJP) at different voltages (5, 10, 12, 15, 17, 20, 25, 30 and 50 V) in control conditions and after incubation with N$^{\omega}$-nitro-L-arginine (L-NNA) (1 mM) and L-NNA (1 mM) + MRS2279 (1 μM). (B) Graphs representing the inhibitory effect of L-NNA (1 mM), and L-NNA + P2Y$_1$ antagonists: MRS2179 (20 μM), MRS2279 (1 μM) and MRS2500 (1 μM) on both the amplitude (top) and duration (bottom) of the EFS-induced IJP. All values represent the mean ± SEM. Significant differences were assessed using two-way ANOVA.

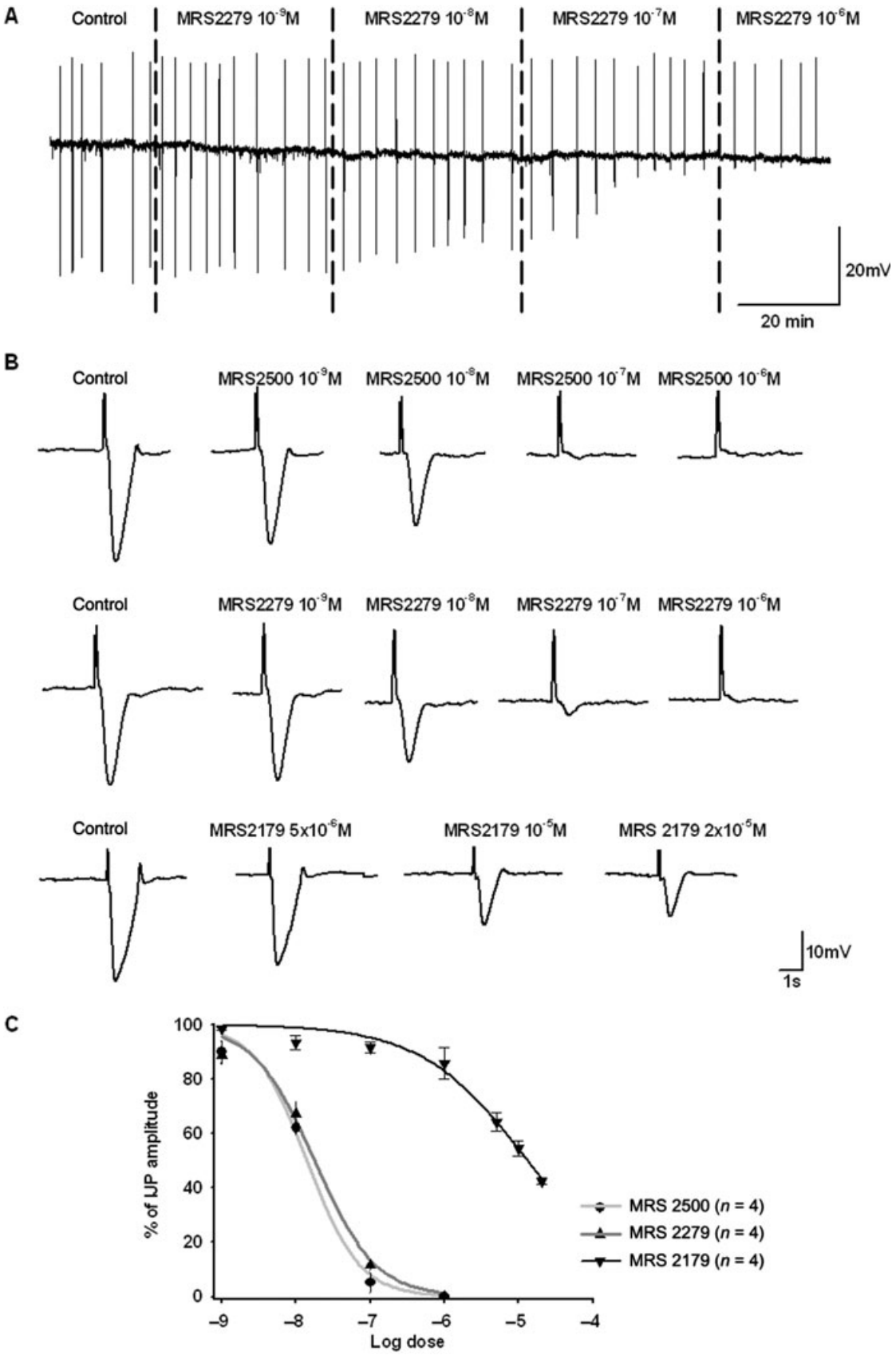

Figure 5 (A,B) Intracellular microelectrode recordings showing the electrical field stimulation-induced supramaximal inhibitory junction potential (IJP) in the presence of N$^{\omega}$-nitro-L-arginine (control) and after cumulative perfusion with the P2Y$_1$ antagonists. (C) Concentration-response curves for the effects of P2Y$_1$ antagonists on the amplitude of the IJP. Note the difference between MRS2179 and MRS2279 or MRS2500.

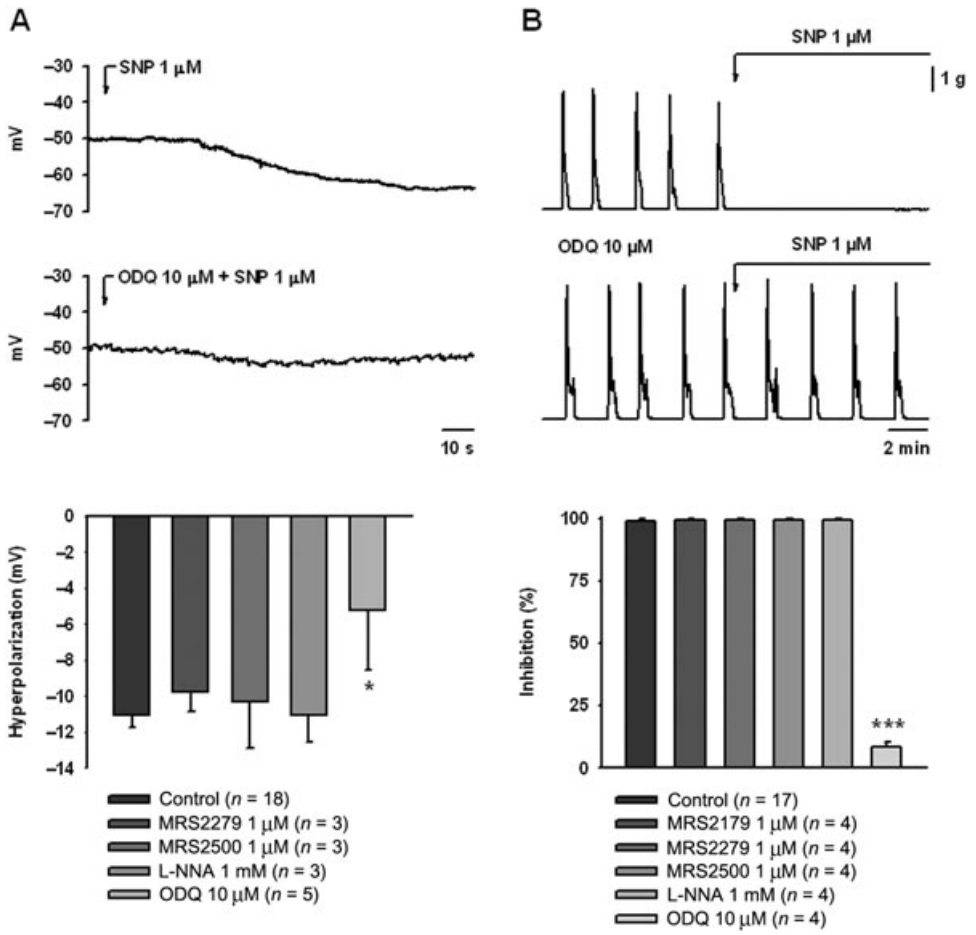

Figure 6 (A) Intracellular microelectrode recordings and histograms showing the hyperpolarization induced by sodium nitroprusside (SNP) (1 μM). oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (10 μM), but not the P2Y$_1$ antagonists, antagonized the hyperpolarization induced by SNP. (B) Inhibition of spontaneous motility by SNP (1 μM). Data were calculated using the following formula: 1 − [area under the curve (AUC) after SNP addition/AUC previous SNP addition] × 100. ODQ (10 μM), but not the P2Y$_1$ antagonists or N$^{\omega}$-nitro-L-arginine (L-NNA), antagonized the inhibitory effect of SNP. All values are mean ± SEM. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$; ***$P < 0.001$, significant difference from control.

antagonists, and the slow component to NOS inhibitors. A combination of NOS inhibitors and P2Y$_1$ antagonists completely blocked the inhibition of spontaneous motility induced by EFS and the off-contraction observed at the end of the stimulus. These results suggest that ATP (or a related purine) and NO are two neurotransmitters that act through P2Y$_1$ receptors and the cGMP pathway respectively. The selective P2Y$_1$ receptor antagonists tested in the present study showed different potency, MRS2500 being greater than MRS2279, which was greater than MRS2179.

The present experiments are a continuation of a previously published study where we demonstrated that the IJPf in the rat colon is mainly sensitive to apamin and suramine, and the IJPs is sensitive to NOS inhibitors (Pluja *et al*., 1999). Suramine is a non-selective purinergic antagonist, and in the present study, we have used newly available pharmacological tools to characterize the co-transmission process.

MRS2179 was the first P2Y$_1$ selective receptor antagonist to be characterized (Camaioni *et al*., 1998). This antagonist has been used in several biological systems involving P2Y$_1$ receptors, such as an inhibitor of platelet aggregation (Baurand *et al*., 2001; Baurand and Gachet, 2003) or nucleotide-mediated relaxation in guinea pig aorta (Kaiser and Buxton, 2002). Recently, two other P2Y$_1$ antagonists, MRS2279 and MRS2500, have become available, and they show comparatively higher affinity and potency to the P2Y$_1$ receptor (Kim *et al*., 2001; Boyer *et al*., 2002; Cattaneo *et al*., 2004). All these compounds are essential pharmacological tools for investigating functions involving P2Y$_1$ receptors (King, 2002). This is the first study in which the selective P2Y$_1$ antagonists MRS2279 and MRS2500 have been used to investigate the IJP and non-nitrergic relaxation in the GI tract. Our results show that all the antagonists were able to inhibit the IJPf, the non-nitrergic relaxation and the off-contraction induced by EFS, though with varying potency. MRS2279 and MRS2500 blocked the IJP in the micromolar range, but higher concentrations (up to 20 μM) of MRS2179 were needed to partially inhibit the IJP. Consistent with this result, P2Y$_1$ antagonists had a different range of potency in the non-nitrergic relaxation induced by EFS, MRS2500 being greater than MRS2279, which was greater than MRS2179. The rank order of antagonist potency is similar to that described in the literature (Cattaneo *et al*., 2004; Von Kugelgen, 2006). The effect of MRS2179 is completely washable, but the effect of MRS2279

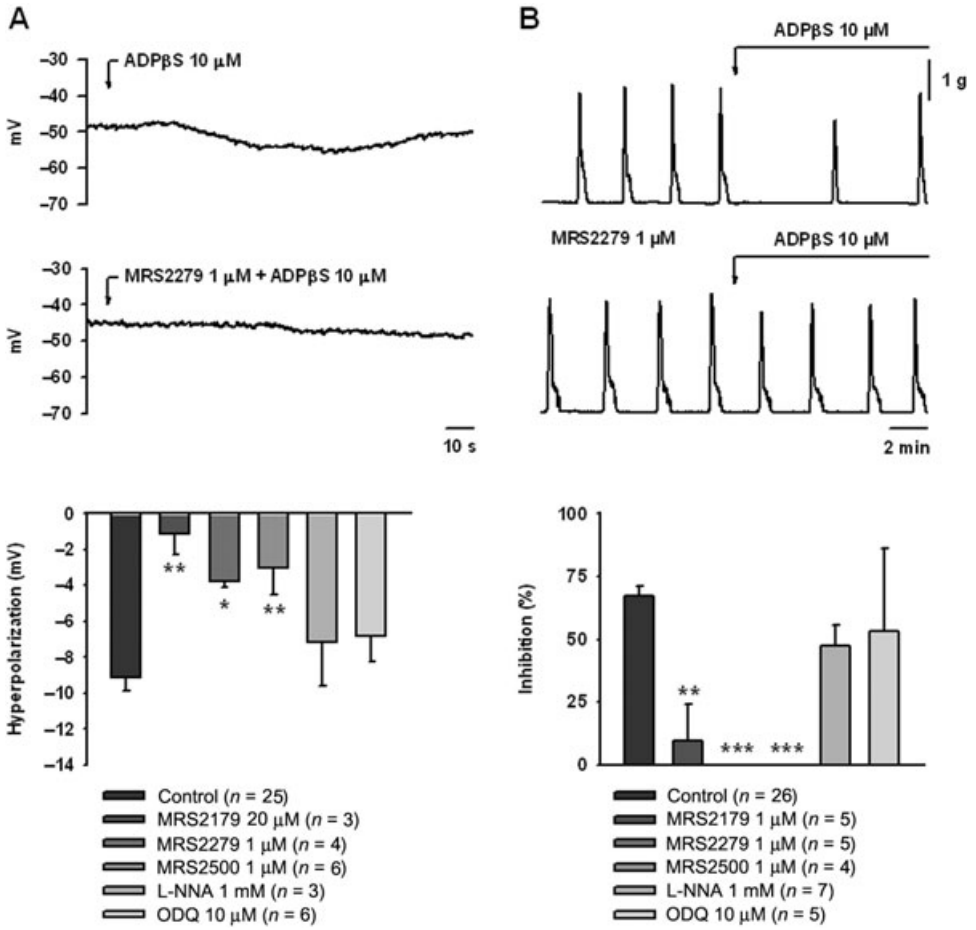

Figure 7 (A) Intracellular microelectrode tracings showing the hyperpolarization induced by ADPβs (10 μM). The hyperpolarization induced by adenosine 5′-O-2-thiodiphosphate (ADPßS) was antagonised by P2Y$_1$ antagonists but not by ODQ (10 mM) or N$^{\omega}$-nitro-L-arginine (L-NNA) 1 mM. (B) Inhibition of spontaneous motility induced by ADPßS (10 μM). Data were calculated using the following formula: 1 − [area under the curve (AUC) after ADPßS addition/AUC previous ADPßS addition] × 100. P2Y$_1$ antagonists, but not L-NNA (1 mM) or ODQ (10 μM), antagonized the inhibition of spontaneous activity. All values are mean ± SEM. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001, significant difference from control.

and MRS2500 is only partially washable, and this might be due to varying sensitivity to ectonucleases (Ravi *et al.*, 2002). Accordingly, MRS2500 has been used as a stable antithrombotic agent *in vivo* (Hechler *et al.*, 2006).

Differences between species might explain present and previously published data. According to the present results and those from other studies (Zizzo *et al.*, 2007; McDonnell *et al.*, 2008), the inhibitory effect of MRS2179 is less potent in rodents than in humans (Gallego *et al.*, 2008a), pigs (Gallego *et al.*, 2008b) or guinea pigs (Wang *et al.*, 2007). In human colon and pig small intestine, the IC$_{50}$ of MRS2179 is close to 1 μM, but in rats, a higher concentration is needed to inhibit EFS-induced relaxation and off-contraction. MRS2279 and MRS2500 have not been used in human colon, but preliminary data from our laboratory show that this antagonist inhibits the human IJPf and relaxation in the nM range. All these data are consistent with a major role of post-junctional P2Y$_1$ receptors in mediating IJPf and non-nitrergic relaxation. The presence of other P2Y receptors in the internal anal sphincter of mice (McDonnell *et al.*, 2008), or, alternatively, a pre-junctional effect of P2Y receptors in the mouse caecum, has been recently postulated (Zizzo *et al.*, 2007). According to the data presented in the present work, further research is needed with more potent P2Y$_1$ antagonists to determine whether the maximal effect has been reached in these tissues. In the rat colon, only P2Y$_1$ and P2Y$_6$ receptors have been immunolocalized in smooth muscle cells (Van Crombruggen *et al.*, 2007). However, MRS antagonists fail to block the rat P2Y$_6$ receptors expressed in human astrocytoma cells (Boyer *et al.*, 1998). P2Y$_1$ receptors are located in enteric neurones in several species (Gao *et al.*, 2006; Gallego *et al.*, 2008b) and mediate slow excitatory postsynaptic potentials in neurones from the guinea-pig submucous plexus (Hu *et al.*, 2003; Monro *et al.*, 2004) and myenteric plexus (Gwynne and Bornstein, 2009). A pre-junctional effect of P2Y$_1$ receptors inhibiting ATP release is possible, but P2Y$_1$ receptors have not been detected in enteric neurones in the rat colon (Van Crombruggen *et al.*, 2007). In a fine, recent study, P2Y$_1$ and P2Y$_{11}$ receptors were shown to mediate the fast and slow relaxation, respectively, in the guinea-pig taenia coli (King and Townsend-Nicholson, 2008). In the guinea pig taenia coli, both P2Y$_1$ and P2Y$_{11}$ receptors are present in smooth muscle cells and α-β meATP activated P2Y$_{11}$ receptors, causing smooth muscle relaxation. However, MRS2179 did not

antagonize calcium increase induced by purinergic activation of human $P2Y_{11}$ receptors transfected in a human astrocytoma cell line (King and Townsend-Nicholson, 2008). In the rat colon, neither have $P2Y_{11}$ receptors been detected in smooth muscle cells, nor in interstitial cells of Cajal, which might participate in neurotransmission (Van Crombruggen *et al*., 2007). All these data are consistent with a major role of $P2Y_1$ receptors in the generation of IJPf and non-nitrergic relaxation. However, the possibility that other extrajunctional P2Y receptors are involved in smooth muscle relaxation needs further research.

It is important to note that blockade of $P2Y_1$ receptors alone does not modify the duration of the IJP nor the inhibition induced by EFS. According to our results, the 'non-purinergic' relaxation is mainly nitrergic. This is consistent with recently published data on the human colon where $P2Y_1$ antagonist inhibited IJPf but not the sustained IJP elicited by electrical pulses (Gallego *et al*., 2008a). In the presence of purinergic blockade, the nitrergic IJP causes smooth muscle hyperpolarization, the membrane potential does not reach the threshold to open calcium channels, and, consequently, spontaneous contractions do not occur. At the end of the stimulus, smooth muscle repolarization probably activates L-type calcium channels, and an off-contraction occurs. In agreement with these results, SNP, an NO donor, causes long-lasting hyperpolarization and smooth muscle relaxation (Pluja *et al*., 1999), which are sensitive to ODQ, but not to $P2Y_1$ receptor antagonists (present work).

In contrast, NO synthase inhibition reduced the IJPs but not IJPf; a partial effect on the inhibition of spontaneous motility was observed in the presence of L-NNA. In this case, the 'non-nitergic' IJP is mainly purinergic through $P2Y_1$ receptors. It is possible that the reduction in the IJP duration would result in smooth muscle cells transiently reaching the threshold to open calcium channels, and this could explain the partial contractions present during EFS. A similar result has been reported in the human colon (Gallego *et al*., 2008a).

In conclusion, the present work demonstrates that in the rat colon, a co-transmission process that involves ATP through $P2Y_1$ receptors, and NO is present. As described in other species, $P2Y_1$ receptors are responsible for the IJPf, and NO is responsible for the IJPs. The rank order of potency for the $P2Y_1$ receptor antagonists in the rat colon is MRS2500 greater than MRS2279, which is greater than MRS2179. It would be interesting to investigate the potencies of these antagonists in human colonic tissue.

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