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Complete title: Different responses of the blockade of the P2Y1 receptor with BPTU in human and porcine intestinal tissues and in cell cultures.

Running title: BPTU antagonism on purinergic neurotransmission.

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Abstract

Background: Gastrointestinal smooth muscle relaxation is accomplished by activation of $P2Y_1$ receptors, therefore this receptor plays an important role in regulation of gut motility. Recently, BPTU was developed as a negative allosteric modulator of the $P2Y_1$ receptor. Accordingly, the aim of this study was to assess the effect of BPTU on purinergic neurotransmission in pig and human gastrointestinal tissues.

Methods: Ca^{2+} imaging in tSA201 cells that express the human $P2Y_1$ receptor, organ bath and microelectrodes in tissues were used to evaluate the effects of BPTU on purinergic responses.

Key results: BPTU concentration-dependently (0.1 and 1 μM) inhibited the rise in intracellular Ca^{2+} evoked by ADP in tSA201 cells. In the pig small intestine, 30μM BPTU reduced the fast-inhibitory junction potential by 80%. Smooth muscle relaxations induced by electrical field stimulation were reduced both in pig ileum (EC_{50}=6 μM) and colon (EC_{50}=35 μM), but high concentrations of BPTU (up to 100μM) had no effect on human colonic muscle. MRS2500 (1μM) abolished all responses. Finally, 10 μM ADPβS inhibited spontaneous motility and this was partially reversed by 30μM BPTU in pig, but not human colonic tissue and abolished by MRS2500 (1μM).

Conclusions & Inferences: BPTU blocks purinergic responses elicited via $P2Y_1$ receptors in cell cultures and in pig gastrointestinal tissue. However, the concentrations needed are higher in pig tissue compared to cell cultures and BPTU was ineffective in human colonic tissue.

Key words: $P2Y_1$ receptors, purinergic response, BPTU, colon, tSA201 cells.

Introduction

G protein-coupled receptors (GPCRs) are involved in many gastrointestinal (GI) mechanisms related to perception, motility and secretion, so GPCR agonists and antagonists are important modulators of GI function. The orthosteric site of the receptor is often highly conserved and consequently, drugs that bind to this site can lack selectivity between different receptor subtypes. One approach to increasing selectivity is to develop pharmacological tools that bind to allosteric sites, which, therefore, can potentiate or inhibit endogenous agonists. Allosteric sites are usually less conserved than the orthosteric site and so offer opportunities for receptor subtype-selective GI modulation [1].

Two main neurotransmitters, nitric oxide (NO) and adenosine triphosphate (ATP), or a related purine, mediate inhibitory neuromuscular transmission in the GI tract. The electrophysiological mechanism responsible for the relaxation is an inhibitory junction potential (IJP), which consists of a fast (IJPf), followed by a sustained (IJPs) component that are mediated by purines and NO, respectively [2 3]. It is well established that $P2Y_1$ receptors (a Class A GPCR) are responsible for nerve-mediated purinergic relaxation in the GI tract. The IJPf and the corresponding purinergic relaxation is absent in Knockout mice that lack $P2Y_1$ receptors [4 5 6]. Furthermore, the $P2Y_1$ receptor orthosteric inhibitors, MRS2179, MRS2279 and MRS2500, all inhibit the IJPf and purine-mediated relaxation of both the small and large human intestine in a concentration-dependent manner [7 8].

Recently, an allosteric inhibitor, 1-(2-(2-(*tert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)- phenyl)urea (BPTU) was developed, which binds to a pocket at the external interface of the $P2Y_1$ receptor with the lipid bilayer, making it the first structurally characterized, selective GPCR ligand that acts entirely outside of the helical bundle [9]. The molecular mechanism of $P2Y_1$ receptor activation and blockade has been characterized in detail [10 11]. A pharmacological study performed in vitro has shown that the inhibitory effects of BPTU and MRS2500 can be

surmountable or unsurmountable depending on the signalling pathway measured, and the agonist used to activate the receptor [12].

All these studies have been performed in cell cultures and therefore, it is mandatory to study these compounds in tissues. In 2016, we reported that BPTU blocked the IJPf and the mechanical purinergic relaxation in colonic muscle strips of rodents [13]. Recently, we demonstrated that both MRS2500 and BPTU blocked the hyperpolarization and relaxation induced by diadenosine tetraphosphate (Ap4A) in the rat colon [14]. Accordingly, the aim of this work was to determine the effects of BPTU first in a large animal model and then in human tissue. Finally, due to possible discrepancies between these results we wanted to compare these actions with mouse colonic tissue and cultured cells in which the human $P2Y_1$ receptor is endogenously expressed.

Materials and methods

Cell culture

tSA201 cells (ECACC Cat# 96121229, RRID:CVCL_2737), a modified HEK293 cell line, were grown and prepared for recording of intracellular Ca^{2+} levels as described previously [15] [16]. Briefly, cells were maintained in 5% CO_2, 95% O_2 in a humidified incubator at 37°C, in Dulbecco's Modified Eagle's Medium (Life Technologies, Paisley, UK), supplemented with 10% foetal calf serum, 1% non-essential amino acids, 1% penicillin (10,000 units mL^{-1}) and streptomycin (10 mg mL^{-1}). Prior to recording, the cells were plated onto 13 mm glass coverslips coated with poly-L-lysine (0.1 mg mL^{-1}) and experiments performed once a confluent monolayer of cells had developed.

Tissue preparation

Mouse Tissue. Eight CD1 mice (male, 8-18 wk old) were housed under controlled conditions: constant temperature (22 ± 2 ºC) and humidity (55 ± 10 %), 12 h light/dark cycle and *ad libitum* access to water and food. Mice were euthanized by cervical dislocation. The colon was quickly removed and placed in carboxygenated (5 % CO_2:95 % O_2) Krebs solution. The mesenteric fat

was removed, the colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing up. The mucosal and submucosal layers were removed, and 1 cm long by 0.4 cm wide strips were cut in a circular direction. The experimental protocol was review and authorised by the Ethical Committee of the Universitat Autònoma de Barcelona.

Pig Tissue. Sixteen Danbred piglets (male, 4-5 wk old, 6-9 kg) were group-housed under conventional conditions in a light (13 h:11 h light/dark cycle) and temperature-controlled (28 ± 2 °C) room. To maintain an optimal temperature, partial floor heating and a heat lamp were provided in the pen. Commercial feed and drinking water were provided *ad libitum*. Euthanasia was performed under intramuscular sedation with Xylazine (2.2 mg kg^{-1} BW; Rompun, Bayer) and Zolazepam - Tiletamine (8 mg kg^{-1} BW; Zoletil 100, Virbac) and carried out by means of intravenous sodium pentobarbital (200 kg^{-1} BW; Dolethal, Vetoquinol S.A.) in the Animal Facility of the Veterinary Faculty (UAB). Pieces of colon (n=16) and ileum (n=16) were used to perform the experiments. Tissues were collected and transported to the laboratory in ice-cold carbogenated Krebs solution. Once in the laboratory, specimens were placed on a dissection dish, and the mucosal and submucosal layers were gently removed. Muscle strips (1 cm long by 0.4 cm wide) were cut in circular orientation. The experimental protocol was review and authorised by the Ethical Committee of the Universitat Autònoma de Barcelona.

Human Tissue. Samples of colon from macroscopically normal regions were obtained from patients (n = 10; 5 woman and 5 men, aged 57–89 years, supplementary figure 1) during colon resections for neoplasm and transported to the laboratory in cold saline buffer. The tissue was placed in Krebs solution on a dissection dish and the mucosal layer was removed. Circular muscle strips (1 cm long by 0.4 cm wide) were cut. The patients of this study provided written, informed consent, and the experimental procedure was approved by the Ethics Committee of the Hospital de la Vall d'Hebron (Barcelona).

Ca^{2+} imaging

Cells were bathed in a buffer comprising (mM): NaCl 122; KCl 5; HEPES 10; KH_2PO_4 0.5; NaH_2PO_4 0.5; $MgCl_2$ 1; glucose 11; $CaCl_2$ 1.8, titrated to pH 7.3 with NaOH. Intracellular Ca^{2+} was monitored using the Ca^{2+}-sensitive fluorescent indicator, Cal-520. Cells on a coverslip were incubated for 1 h at 37°C in the dark in buffer containing Cal-520-AM ester (5 µM) and Pluronic™ F-127 (0.05% w/v in DMSO) (Life Technologies, Paisley, UK). The coverslip was then placed vertically in the recording chamber of a Perkin Elmer LS50B luminescence spectrophotometer and the cells superfused continuously with buffer, applied under gravity at 4 ml min^{-1} and room temperature. Cal-520 fluorescence, measured as arbitrary units (AU) in a population of cells, was sampled at 10 Hz following stimulation at 490 ± 15 nm and the emission recorded at 525 ± 15 nm using FL Winlab software (V4.00.02). Resting Ca^{2+} levels were stable over the course of the experiment. Agonists were added in the superfusate until the response reached a peak (60-90 s) at 10 min intervals. For each drug addition, the peak response amplitude was determined. The following drugs were used in the experimental protocol: Adenosine 5'-diphosphate sodium salt (ADP) (Sigma-Aldrich Co, Gillingham, Dorset, UK) which was dissolved in deionised water and BPTU (Tocris, Bristol, UK) that was dissolved in DMSO. 0.1% DMSO has no effect on Ca^{2+} levels or nucleotide evoked responses in tSA201 cells (Kennedy, *unpublished observations*).

All coverslips of tSA201 cells were first exposed to ADP (10 µM) twice to confirm cell viability. To determine the potency of ADP, concentration-response curves (CRC) were then generated by superfusing cells with increasing concentrations of ADP. The data were normalised by calculating each response in AU as a percentage of the response to ADP 10 µM within the CRC. When characterising the actions of BPTU, only one concentration of ADP and BPTU were applied to each coverslip of cells. Cells were first exposed to ADP 10 µM twice, then the test concentration of ADP was applied three times. This protocol evoked reproducible responses. BPTU 100 nM or 1 µM was then applied to the cells for 5 min, before co-administration with ADP. The responses to ADP in AU were normalised by expressing them as a percentage of the amplitude of the second response to ADP 10 µM.

Mechanical experiments

Ileum and colon circularly oriented muscle strips were mounted in a 10 mL organ bath filled with Krebs solution maintained at 37 ± 1 °C. A tension of 0.5, 1 and 4 g were applied to mouse, pig and human strips, respectively and they were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic contractions (SPC). In order to increase the spontaneous mechanical activity, pig and human tissues were incubated with carbachol (1 μM). An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using DATAWIN1 software (Panlab, Barcelona, Spain) coupled to an ISC-16 analog-to-digital card installed in a PC. Electrical field stimulation (EFS) was applied through two platinum electrodes placed on the support holding the tissue. Colonic preparations were stimulated at 5 Hz, 0.4 ms pulse duration and 10 to 30 V for 1 to 2 min. The area under the curve (AUC) (g min^{-1}) of contractions from the baseline was measured to estimate mechanical activity before and after drug addition or before and during EFS. In order to normalize mechanical data, responses to drugs and EFS were expressed as a percentage of the basal AUC using the following formula: 1 – (AUC during EFS or after drug incubation / AUC previous to EFS or drug addition) thus 0% represents complete cessation of spontaneous motility and 100% no change compared to basal activity.

Electrophysiological experiments

Electrophysiological experiments were performed with pig ileum strips dissected parallel to the circular muscle and pinned in a Sylgard- coated recording chamber. The tissue was continuously superfused with carboxygenated Krebs solution maintained at 37 ± 1 °C and allowed to equilibrate for approximately 1 h before experiments were undertaken. Phentolamine, atropine and propranolol (all at 1 μM) were added to create non-adrenergic, non-cholinergic (NANC) conditions. To obtain stable impalements, tissues were superfused with nifedipine (1 μM) to abolish mechanical activity. L-NNA (1 mM) was also added to the Krebs solution to block nitrergic

neurotransmission. Circular muscle cells were impaled with single, sharp glass microelectrodes filled with KCl (3 M) and with a tip resistance of 40 – 60 MΩ in order to record membrane potential responses to EFS and drugs. Membrane potential was measured using a standard electrometer, Duo773 (WPI, Sarasota, FL, USA). Recordings were displayed on an oscilloscope, 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) with Power- Lab 4/30 system and Chart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). Intramuscular neurons were stimulated by EFS using a pair of silver chloride electrodes, one on each side of the preparation. IJPs were elicited by EFS (0.3 ms pulse width, supramaximal voltage). The amplitude (mV) of the IJPf was calculated by measuring the difference between the maximal hyperpolarization and the resting membrane potential (rmp).

Solutions and drugs

The composition of the Krebs solution used in the mechanical and electrophysiological experiments was as follows (mM): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$, bubbled with a mixture of 5% CO_2:95% O_2 (pH 7.4). The following drugs were used: nifedipine, Nω-nitro- L-arginine (L-NNA), phentolamine, atropine sulphate, propranolol, adenosine 5'-[β-thio] diphosphate trilitium salt (ADPβS) (Sigma Chemicals, St. Louis, MO, USA), byciclo [3.1.0] hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500), 2'-deoxy-N6-methyl adenosine 3',5'-diphosphate tetraammonium salt (MRS2179), (2-Hydroxyethyl)trimethylammonium chloride carbamate (Carbachol) (Tocris, Bristol, UK), BPTU (Merck Millipore, Darmstadt, Germany). Stock solutions were made by dissolving drugs in distilled water, except for nifedipine, which was dissolved in ethanol (96%) (<0.001% v/v), BPTU, which was dissolved in DMSO and L-NNA which required sonication to be dissolved in Krebs solution.

Data analysis and statistics

ADP CRC in Ca^{+2} imaging was fitted to the data by logistic (Hill equation), nonlinear regression

analysis and EC_{50} and maximum values calculated. EC_{50} values for BPTU, MRS2500 and MRS2179 on EFS-induced inhibition of spontaneous motility in pig ileum and colon were derived in the same way. Responses to drugs and EFS in mechanical experiments were expressed as a percentage of the basal AUC of contractions. As only one concentration of ADP and BPTU were applied to each coverslip of cells, Student's paired *t* test was used to evaluate the differences in the rise in intracellular Ca^{2+} induced by ADP in the absence and presence of BPTU. The effect of drugs on IJP and EFS or drug induced inhibition of spontaneous motility was evaluated by One way ANOVA followed by a Bonferroni's post hoc test. Data are expressed as mean ± SEM and considered significantly different when $P<0.05$. n values indicate the number of samples from different cell cultures or pig or human tissues. Statistical analysis and curve fit were performed with GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effect of BPTU on ADP-induced Ca^{2+} release

tSA201 cells, a modified Human Embryonic Kidney 293 cell line that expresses native $P2Y_1$ receptors, were used to determine the actions of BPTU at human $P2Y_1$ receptors. ADP evoked a concentration-dependent rise in intracellular Ca^{2+}, with an EC_{50} of 2.1 µM (1.5-2.9 µM 95% confidence limits) (n=4). Initial experiments with BPTU indicated that it is a non-competitive and irreversible antagonist, therefore, its actions were determined against three concentrations of ADP that were towards the bottom (1 µM), middle (3 µM) and the top (10 µM) of the quasi-linear portion of the ADP CRC. Preincubation with BPTU 100 nM had no effect *per se* on intracellular Ca^{2+} levels, but significantly decreased the responses to 1 µM ADP by 91.3 ± 5.3%, to 3 µM ADP by 76.6 ± 3.2% and 10 µM ADP by 53.1 ± 1.9% (Figure 1A) ($P<0.001$ each). A ten-fold higher concentration of BPTU, 1 µM, also had no effect of its own on intracellular Ca^{2+} levels, but abolished the response to ADP 1 µM and further reduced the responses to 3 and 10 µM by 90.0 ± 6.5% ($P<0.001$) and 74.3 ± 4.5% ($P<0.0001$), respectively (Figure 1B). There was no

reversal of the inhibition by both concentrations of BPTU, even after up to 30 min washing with drug-free buffer (Figure 1C).

Effect of BPTU on EFS-induced relaxation

In order to record constant spontaneous contractions, tissues were incubated with carbachol (1 μM). In pig ileum and colonic strips, EFS caused a cessation of spontaneous contractions (Figure 2A,E,F) that was partially reversed by L-NNA (1 mM) (by 38.7 ± 14.9 %-ileum and 32.8 ± 8.5 %-colon) (Figure 2B,E,F). When increasing concentrations of BPTU (1, 10 and 30 μM) were then added in the continued presence of L-NNA, it caused a further, concentration-dependent reduction in the inhibitory responses elicited by EFS (Figure 2C,E,F). However, subsequent addition of MRS2500 (1 μM) was required to achieve complete reversal of the EFS-induced relaxation (Figure 2D,E,F). The EC_{50} for BPTU was higher in the pig colon compared with the ileum ($P<0.05$), being 35 μM and 6 μM, respectively (Table 1). Since the concentration of BPTU needed to block the inhibition of spontaneous contractions in pig tissues was higher compared to the results previously obtained in rodents [13], the effect of BPTU was also determined in the mouse colon. Using the same protocol as described above, BPTU reversed the EFS response at lower concentrations than those used in pig tissues (Figure 2G, Table 1).

Next, the potencies of MRS2500 and MRS2179, another orthosteric $P2Y_1$ antagonist, were determined in pig tissues following the same protocol. Both blocked EFS-induced responses in a concentration-dependent manner, though higher concentrations of MRS2179 were needed compared with MRS2500 (pig ileum $P<0.0001$, pig colon $P<0.05$) (Figure 3, Table 1).

In human colon, L-NNA (1 mM) also produced a partial reduction of the EFS-induced relaxation (34.7 ± 10.4 %). In contrast with the results obtained in pig tissues, BPTU (0.01-100 μM) did not significantly reverse EFS-induced inhibitory responses ($P>0.05$), but subsequent addition of MRS2500 (1 μM) produced complete reversal ($P<0.0001$) (Figure 4), consistent with our previously published results [8] (Table 1).

Effect of BPTU on IJPf

In previous experiments we showed that the IJPf was reduced by BPTU in a concentration-dependent manner in colonic tissues [13]. Accordingly, we determined the effect of BPTU on the IJPf in pig small intestine. Slow waves (sw) had an amplitude of 8.0 ± 1.5 mV and a frequency of 9.8 ± 0.3 contractions per minute and the rmp was -62.1± 1.3 mV. However, sw and rmp were not affected by BPTU. In the presence of L-NNA (1 mM), EFS with a single pulse at supramaximal voltages elicited a prominent IJPf that reached a mean peak amplitude of 12.8 ± 2.0 mV (Figure 5A,D). At the lowest concentrations tested, (1 and 10 µM), BPTU did not significantly modify the amplitude of the IJPf ($P>0.05$), but increasing the concentration of BPTU to 30 µM, significantly reduced the IJPf amplitude to 2.7±1.1 mV ($P<0.0001$) (Figure 5B,D). Subsequent coadministration of MRS2500 (1 µM) produced complete blockade of the response ($P<0.0001$) (Figure 5C). These results showed that a higher concentration of BPTU was needed to block purinergic responses compared to MRS2500.

Effect of BPTU on agonists targeting the $P2Y_1$ receptor

Finally, the effects of BPTU on relaxations induced by $P2Y_1$ receptor agonists were determined. In the presence of L-NNA (1 mM), ADPßS (10 µM) significantly reduced spontaneous contractions of pig ileum (Figure 6A,D) and colon (Figure 6B,E) to 17.4 ± 8.5% ($P<0.0001$) and 25.5 ± 9.8% ($P<0.001$) of control, respectively. A similar reduction was also observed in human colon (24.6 ± 9.1% of control) (Figure 6C,F). BPTU (30 µM), partially reversed the ADPßS response in pig ileum ($P<0.01$; 68.3 ± 11.1% of control) and colon ($P<0.05$; 70.1 ± 12.4% of control), but had no effect in human colon, ($P>0.05$; 29.7 ± 10.9% of control) (Figure 6).

Discussion

It is well established that NO and ATP or a related purine, act as cotransmitters and in a complementary manner, via guanylyl cyclase and $P2Y_1$ receptors respectively, to produce nerve-mediated muscle IJPs and relaxation in the GI tract. The rapid IJPf evoked by $P2Y_1$ receptor

stimulation is transient during continuous stimulation, whereas NO-mediated slow IJPs summate. These lead to a time-dependent, transient purinergic relaxation followed by a sustained nitrergic relaxation. In this study, we showed that BPTU inhibits the purinergic responses in the mouse and pig intestine and also that it is much more potent in the former. Interestingly, although BPTU strongly reduces purine-mediated responses in human cells that express the $P2Y_1$ receptor, BPTU had no effect on responses mediated by $P2Y_1$ receptors in human colonic muscle. Thus the actions of BPTU in the GI tract appear to vary with the species studied.

In the present study we showed that 30 μM BPTU reduced the IJPf in the pig small intestine by about 60-70%, whereas MRS2500 totally blocked the IJPf at 1 μM. However, a lower concentration of BPTU was needed to reduce the IJPf, both in mice (EC_{50} = 0.06 μM) and as we previously reported, rats (EC_{50} = 0.3 μM) [13]. We also found that under the same experimental conditions BPTU blocked the purinergic relaxation, but the concentration needed to reduce the response by 50% was higher in pig tissue compared to the values previously reported in rodents (see table 1) [13]. Due to these differences, we repeated the experiments with mouse tissue and we obtained similar results to those previously reported [13]. All together these experiments demonstrate that sub-micomolar concentrations of BPTU block both electrophysiological and mechanical responses in rodents, but concentrations in the micromolar range are needed to block responses in pig intestinal tissue.

Next, we determined the effect of BPTU on human colonic tissue. Consistent with the electrophysiological profile, L-NNA only partially reduced the neurogenic inhibition of spontaneous motility. Due to rundown of the IJPf during continuous stimulation, the purinergic component of relaxation was larger at the beginning compared to the end of the stimulus (see figure 4B). Unexpectedly, even at high concentrations, BPTU did not block this response, whereas subsequent addition of MRS2500 1 μM abolished it (see Figure 4D). Higher

concentrations of BPTU could not be used since the vehicle, DMSO, strongly reduced spontaneous motility. Moreover, the time of incubation usually achieved in this experiment is about 20 min for each concentration. In some experiments, we increased it to about 45 min, but the result was the same (not shown). In order to confirm that BPTU does act as an antagonist at human $P2Y_1$ receptors [12], we investigated the ability of BPTU to block $P2Y_1$ receptor-mediated release of Ca^{2+} in human tSA201 cells. As expected BPTU (100nM and 1 μM) reduced the Ca^{2+} release induced by ADP (1 to 10μM).

In this study ADPßS strongly inhibited spontaneous contractions in all tissues tested and the response was blocked in all cases by MRS2500, as reported previously [17]. However, BPTU 30μM only partially reduced the response in pig tissues and had no effect in human tissue. These data suggest that BPTU is able to reduce $P2Y_1$ responses in pig, but not human tissues. MRS2500 and ADPßS were, however, effective in all tissues. One possible explanation is that whilst MRS2500 and ADPßS are water-soluble nucleotides, BPTU is a hydrophobic non-nucleotide and it may be more difficult for it to penetrate thicker (pig or human) than thinner (rodents) tissues, which would be consistent with its relative potency between the tissues studied. BPTU binds to the receptor in a shallow binding pocket on the external interface of TM1–TM3, within the lipid bilayer, which accommodates the ligand mainly through hydrophobic interactions [18]. Efforts to design less hydrophobic analogues of BPTU resulted in a decrease in the binding affinity [18]. It will be important to bear the lipophilicity and reduced activity of BPTU as potential limitations for future studies on human tissues.

In conclusion, in this study we show that BPTU is a $P2Y_1$ antagonist that reduces purinergic responses in GI tissues from mice and pigs and in a human cell line. However, in colonic human tissue BPTU was ineffective. Accordingly, BPTU is an excellent pharmacological tool to better characterize pharmacological blockade of receptors expressed in cell cultures or in isolated

tissues where diffusion of the drug is not an issue, but there are limitations to its capacity to block purinergic responses in thicker tissues or possibly even in individuals.

AUTORS CONTRIBUTION

ST and MJ performed functional experiments, analysed the recordings and interpreted the data. MRC and CK designed cell culture experiments, JM and LCM conducted cell culture experiments, JM, LCM, MRC and CK performed data analysis and interpreted the data. CB, LR and AA selected the patients and coordinated the samples. PV, AA, CK and MJ contributed to the discussion of the manuscript. ST, CK and MJ drafted the manuscript. All the authors read and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Tables

Table 1. Pharmacological data of the blockade of purinergic response. EC_{50} of MRS2179, MRS2500 and BPTU in preparations from mouse, rat, pig and human.

Species/tissue	**MRS2179 (EC_{50})**	**MRS2500 (EC_{50})**	**BPTU(EC_{50})**
Mouse Colon	----------	0.025µM[13]	0.3µM[13] (present work)
Rat Colon	3.5µM[19]	0.016µM[19]	0.5µM[13]
Pig Colon	0.15µM	0.054µM	35µM
Pig Small Intestine	0.7µM[3]	0.06µM	6µM
Human Colon	0.87µM[2]	0.088µM[8]	----------

Supplementary table 1. Age, sex and tissue localization of the patients.

Age	**Sex**	**Tissue localization**
76 years	Woman	Descending (left) colon
89 years	Woman	Ascending (right) colon
57 years	Men	Ascending (right) colon
62 years	Men	Transverse colon
73 years	Woman	Sigmoid colon
82 years	Men	Sigmoid colon
59 years	Men	Ascending (right) colon
78 years	Men	Ascending (right) colon
74 years	Woman	Sigmoid colon
57 years	Woman	Ascending (right) colon

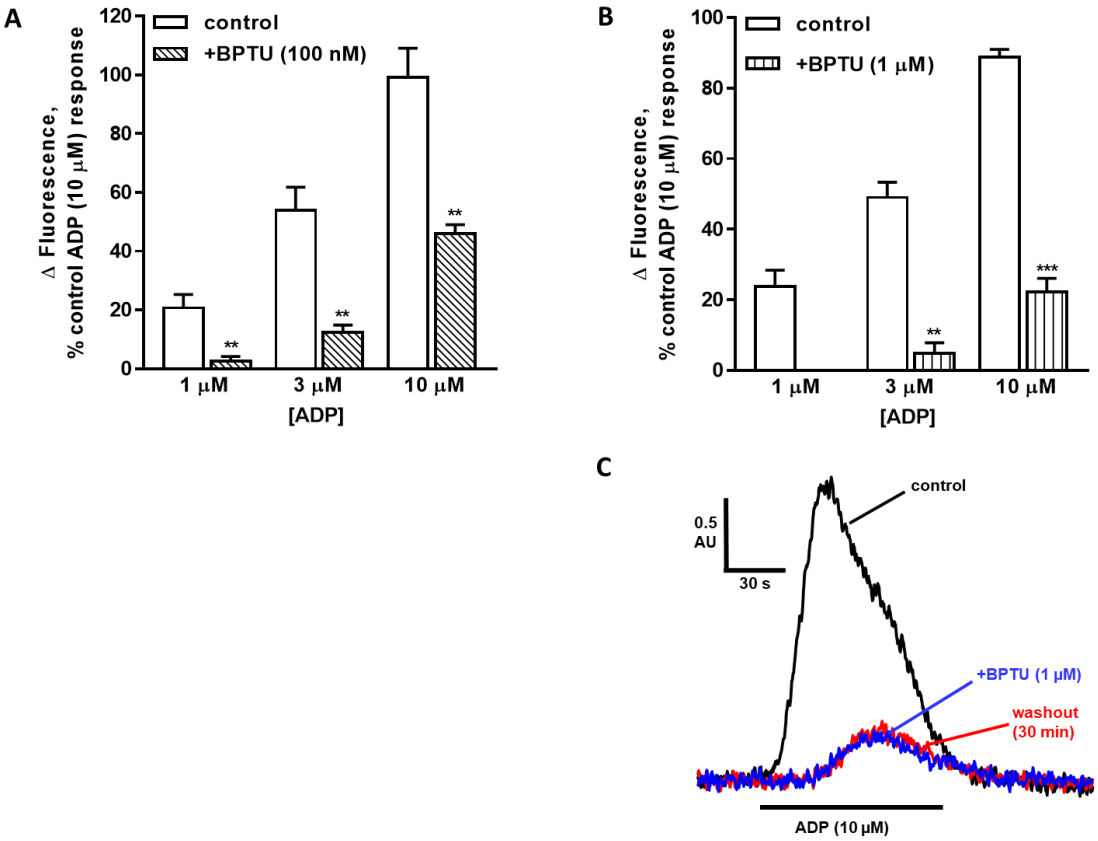

Figure 1. BPTU inhibits human $P2Y_1$ signalling tSA201 cells. (A-B) Peak amplitude of responses evoked by ADP (1, 3, 10 μM) in the absence and presence of BPTU 100 nM and 1 μM, respectively (n=5). (C) Superimposed traces showing changes in Cal-520 fluorescence evoked by superfusion of cells with ADP 10 μM, as indicated by the horizontal bar, before (black), during (blue) and after (red) incubation with BPTU 10 μM. All are from the same population of cells. The data are expressed as a percentage of the response to ADP 10 μM obtained at the start of the experiment. Paired Student's *t* test, **P<0.001 and ***P<0.0001 for responses in the presence of BPTU compared to in its absence on the same coverslip of cells. Histograms represent mean ± SEM.

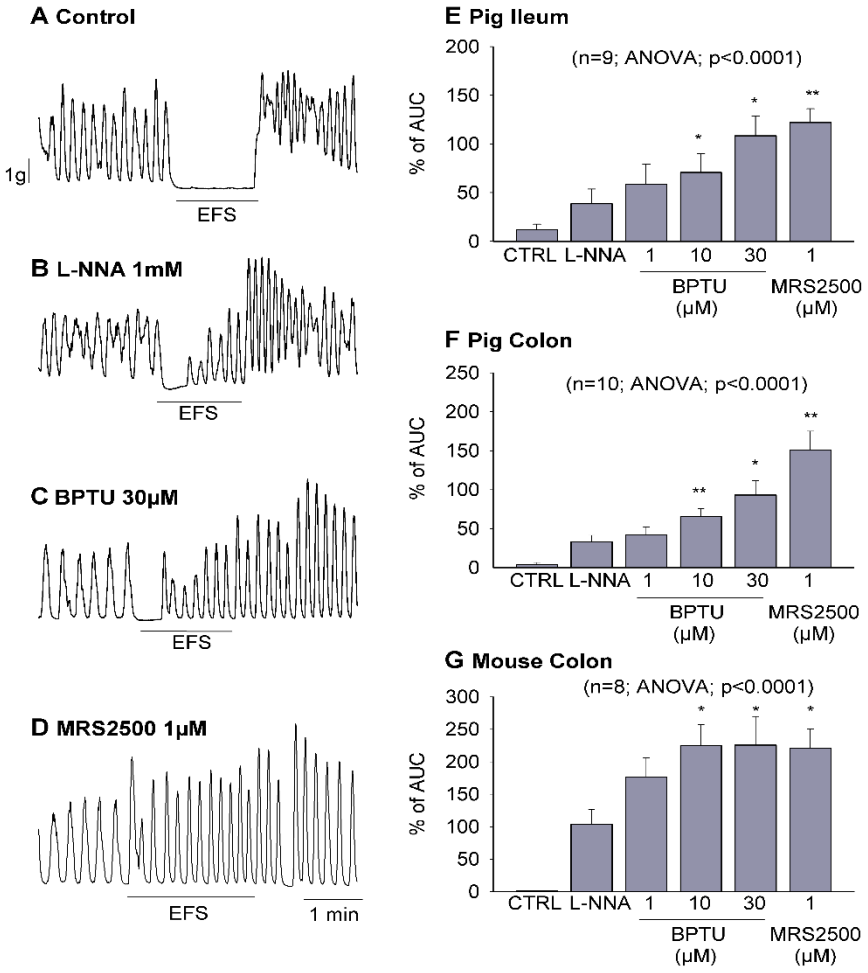

Figure 2. BPTU effect on the inhibition of spontaneous motility induced by EFS in pig and mouse tissue. (left; A-D) Recordings of colonic pig preparations. (right; E-G) Histograms of pig ileum, pig colon and mouse colon tissues showing the effect of $P2Y_1$ blockers in 2 min EFS (5 Hz, 50 V and a pulse duration of 0.4 ms) response in control conditions and in the presence of L-NNA, BPTU 1, 10 and 30 µM and MRS2500 1 µM. Data were compared to basal AUC. One way ANOVA test followed by Bonferroni's post hoc test, *P<0.05 and **P<0.01 for responses in the presence of different concentrations of BPTU compared to in its absence. Histogram represent mean ± SEM. n value are shown at the top of the graph.

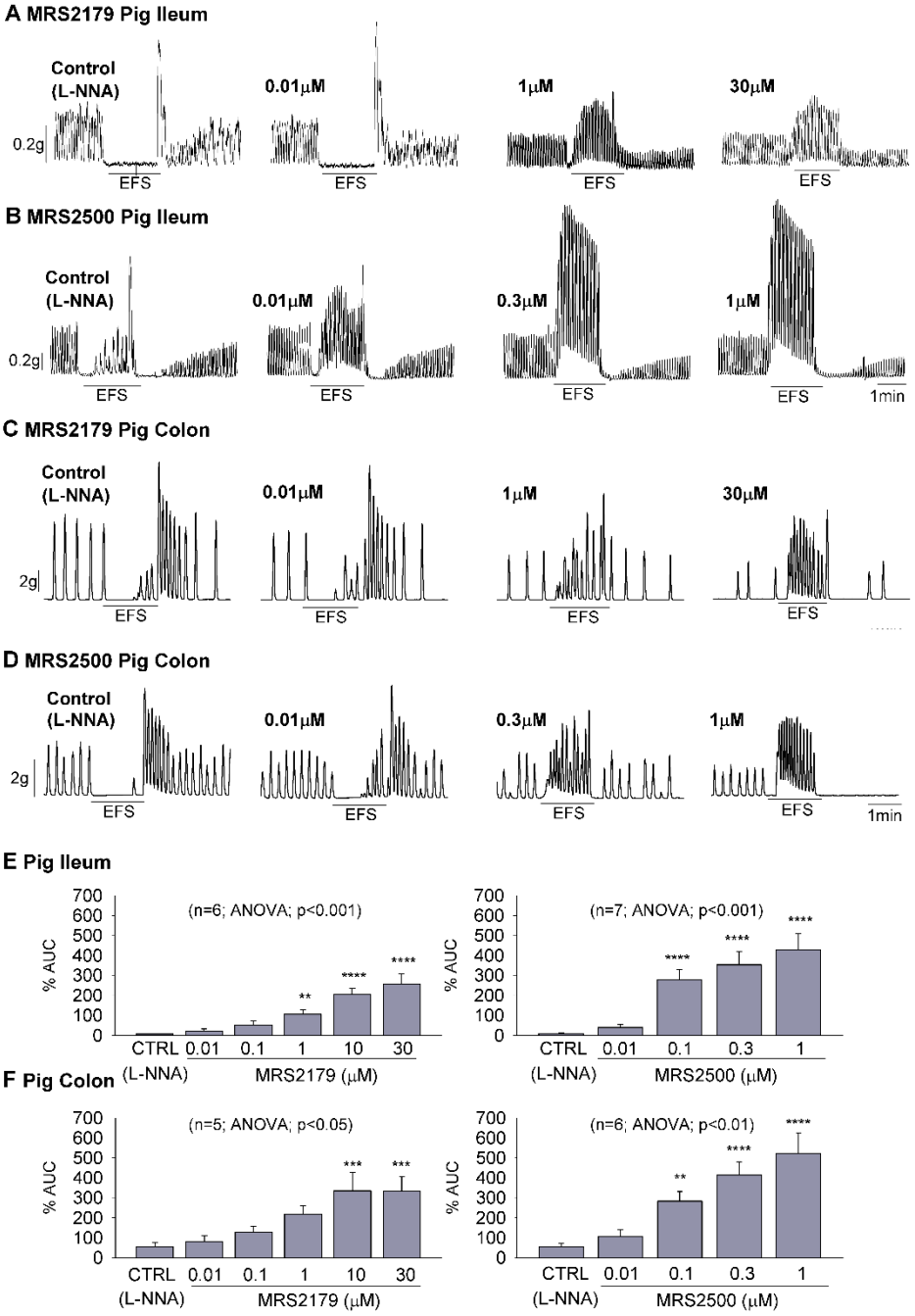

Figure 3. Comparison of the potency of MRS2179 and MRS2500 in the pig ileum and colon. (A-D) Recordings of ileum pig preparations (A, B) and pig colonic strips (C, D) showing the effect of MRS2179 and MRS2500. (E-F) Histograms showing a higher potency of MRS2500 compared to MRS2179. Data were compared to basal AUC. One way ANOVA test followed by Bonferroni's post hoc test, *P<0.05 and **P<0.01 for responses in the presence of different concentrations of MRS2179 and MRS2500 compared to in its absence. Histogram represent mean ± SEM. n value are shown at the top of the graph.

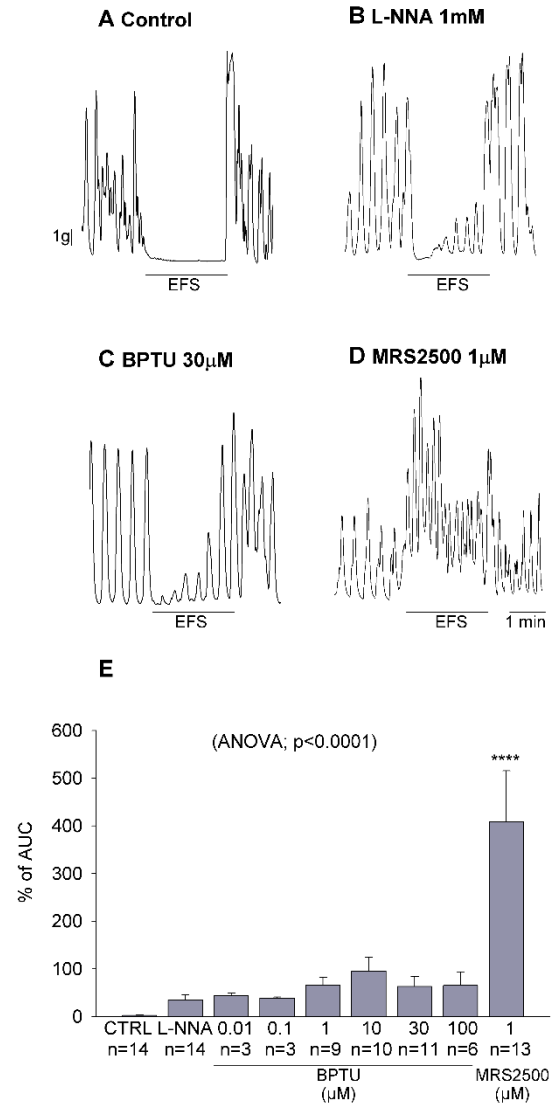

Figure 4. BPTU effect on purinergic relaxation in human colon. (top; A-D) Mechanical recordings and (E) histogram showing 2 min EFS (5Hz, 50V and a pulse duration of 0.4 ms) in the presence of L-NNA 1 mM (B), BPTU 30 μM (C) and MRS2500 1 μM (D). Only MRS2500 1 μM blocked the EFS-induced inhibitory response. Data were compared to basal AUC. One way ANOVA test followed by Bonferroni's post hoc test, P>0.05 for responses in the presence of different concentrations of BPTU and ****P<0.0001 for MRS2500 1μM compared to in its absence. Histogram represent mean ± SEM. n for each group value are shown at the bottom of the graph.

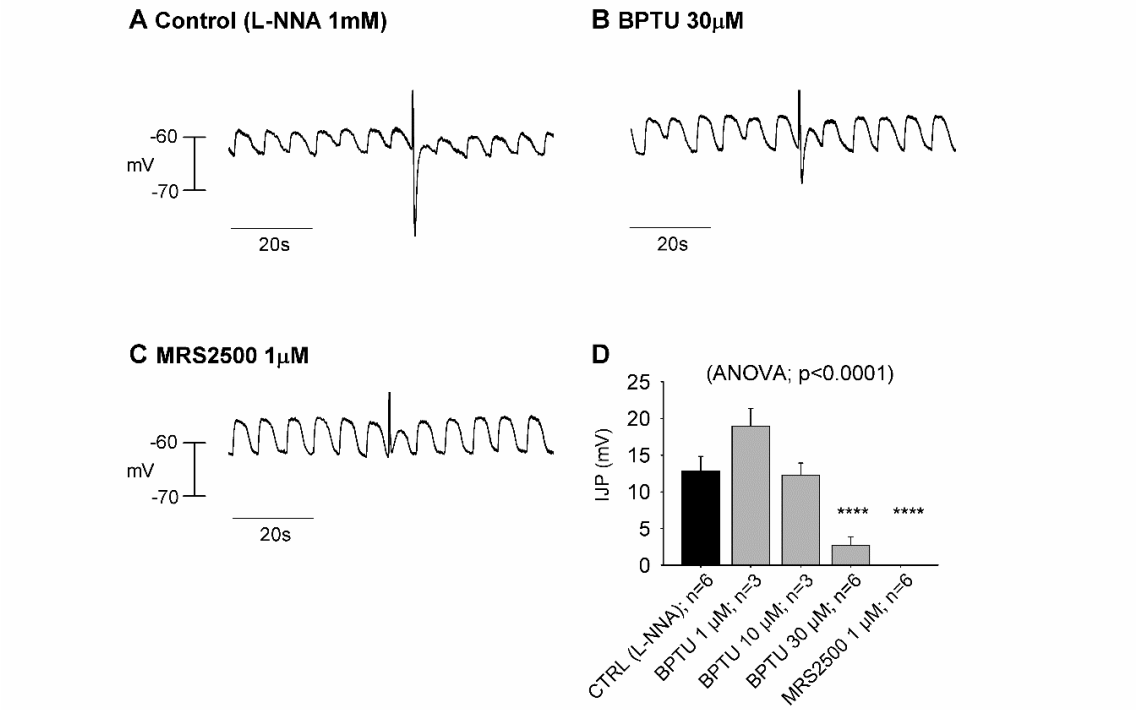

Figure 5. Effect of BPTU on IJPf in the pig ileum. (A-C) Pig ileum recordings showing EFS in control conditions (A), in the presence of BPTU 30μM (B) and after MRS2500 1 μM addition (C). (D) Histogram showing the effect of 1, 10 and 30 μM BPTU on IJPf. Notice a reduction of the amplitude of the IJPf after adding BPTU 30 μM. One way ANOVA test followed by Bonferroni's post hoc test, P>0.05 for responses in the presence of BPTU 1 and 10 μM and ***P<0.001 for BPTU 30 μM compared to in its absence. Histogram represent mean ± SEM. n value is shown at the top of the graph.

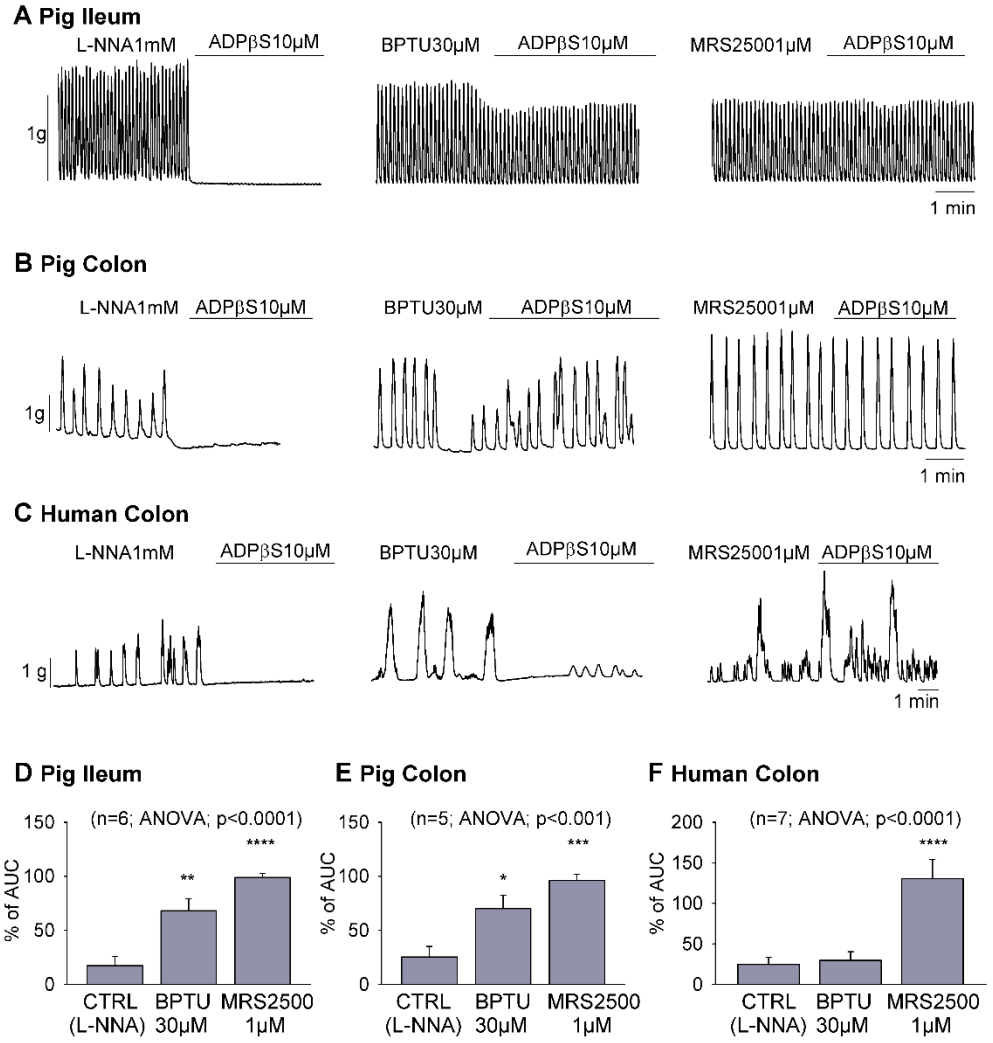

Figure 6. Effect of ADPβS in pig and human preparations. (top; A-C) Mechanical recordings and (bottom; D-F) histograms showing the effect of ADPβS 10 μM in control conditions and in the presence of BPTU 30 μM and MRS2500 1 μM. Notice the inhibitory effect of ADPβS 10 μM compared to basal contractility. BPTU 30 μM only reduced the inhibitory effect of ADPβS in pig tissues and the response was completely blocked by MRS2500 1 μM in all the preparations. Data were compared to basal AUC. One way ANOVA test followed by Bonferroni's post hoc test, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for responses in the presence of BPTU 30 μM and MRS2500 1μM compared to in its absence. Histogram represent mean ± SEM. n value is shown at the top of the graph.