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Preliminary communication

Constitutional isomers of Reactive Blue 2 – selective P2Y-receptor antagonists?

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Dedicated to Professor Dr. Ernst Mutschler on the occasion of the 70th anniversary of his birthday.

Abstract

The anthraquinone derivative Reactive Blue 2 (RB 2) is one of the most widely used P2-receptor antagonists, still claimed to be P2Y-selective. RB 2 is defined as a mixture of two constitutional isomers and commercially available in different identity and purity. A sample of RB 2, offered for sale by RBI, purchased from Biotrend, Köln, Germany, was chromatographically purified and identified by ^{1}H- and ^{13}C-NMR studies as a 35:65 mixture of the terminal ring F *meta* and *para* constitutional isomers. The two constitutional isomers of RB 2 were synthesised and tested alongside with the *ortho* isomer Cibacron Blue 3GA (CB 3GA) on contractions of the rat vas deferens (RVD) elicited by α,β-methylene ATP (α,β-MeATP), mediated by $P2X_1$-receptors, and relaxations of the carbachol-precontracted guinea pig taenia coli elicited by adenosine 5′-*O*-(2-thiophosphate) (ADPβS), mediated by $P2Y_1$-like-receptors. All compounds inhibited the α,β-MeATP induced contraction of the RVD and the ADPβS induced relaxation of the carbachol precontracted guinea-pig taenia coli. The IC_{50}-values at $P2X_1$-R were 9.1 μM for CB 3GA, 28.4 μM for RB 2, 19.7 μM for RB 2 *meta*, and 35.5 μM for RB 2 *para*. The IC_{50}-values at $P2Y_1$-like-R were 17.4 μM for CB 3GA, 7.7 μM for RB 2, 12.0 μM for RB 2 *meta*, and 2.6 μM for RB 2 *para*. The results clearly show that neither RB 2 as a mixture nor the pure *ortho* and *meta* isomer are $P2Y_1$-like- versus $P2X_1$-selective antagonists. In contrast the pure *para*-isomer of RB 2 is a moderately $P2Y_1$-like- versus $P2X_1$-selective antagonist.

Keywords: Reactive Blue 2; Cibacron Blue 3GA; P2-receptor antagonist; Rat vas deferens; Guinea-pig taenia coli; $P2X_1$-receptor; $P2Y_1$-like-receptor

1. Introduction

P2-Receptors (P2-Rs) with the ligand gated ion channel P2X-receptor- and the G-protein coupled P2Y-receptor-classes are of growing interest not only in experimental pharmacology but also as new targets in drug research [1–4]. Therefore, there is great demand for selective and potent P2-receptor antagonists. Reactive Blue 2 (RB 2) [5,6] is one of the most widely used P2-receptor antagonists, still claimed to be P2Y-selective [7–9], although there is now a number of results indicating that it does not discriminate between P2X-R and P2Y-R subtypes [10–18]. Ever since the commercially available dye RB 2 has been used as a tool in purinoceptor research, there was some doubt about its identity and purity [18–23], both of which are prerequisites for reliable receptor characterisation and subdifferentiation.

RB 2 belongs to the anthraquinone–chlorotriazinyl reactive dyes, which formerly were used in textile industry and later became important as ligands in affinity chromatography for purification of enzymes

Abbreviations: C.I., colour index; RB 2, Reactive Blue 2; CB 3GA, Cibacron Blue 3GA; α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; ADPβS, adenosine 5′-*O*-(2-thiophosphate); RVD, rat vas deferens; GPTC, guinea-pig taenia coli; 2D-NMR, two dimensional nuclear magnetic resonance; COSY, Correlation Spectroscopy; HETCOR, heteronuclear correlation; NOESY, Nuclear Overhauser Enhancement Spectroscopy; CC, column chromatography; PIRPHPLC, paired ion reversed phase high performance liquid chromatography.

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and biopolymers such as interferons and albumins [24,25]. These dyes contain a unique combination of ionic and hydrophobic sites. Yet, as commercial preparations they are of highly heterogeneous nature and varied in their actual chemical composition of the major components not only from one manufacturer to the other but also from batch to batch [26–29]. From the 1970s these dyes have also been applied as P2-receptor research tools, since they display a certain degree of antagonistic properties. But reliable receptor research is only warranted with ligands of structural homogeneity and unequivocal designation.

The structure of RB 2 (see Fig. 1) was claimed to be a ring F *meta*/*para* sulphonate mixture [30]. This structure has been assigned the dye constitution Index number 61 211 [31] and is found under the CAS Registry No.: 12236-82-7. For a time the so defined mixture has also been offered for sale under the name of Cibacron Blue F3GA. But Hanggi and Carr found out that of three commercial preparations of Cibacron Blue F3GA only one (Pierce) contained the two constitutional isomers and the content did not exceed 10% of the total mixture. Instead they identified the dichlorotriazinyl analogue of Cibacron blue as the major constituent of all preparations [26]. Their identification was done by stepwise synthesis of the two pure *meta* and *para* isomers and comparative thin layer chromatography (TLC) and paired-ion RP-HPLC. The authors did not provide a complete NMR-spectroscopic characterisation, because of the difficulty in exactly interpreting details of NMR data and of variations from run to run, since strong concentration and pH dependent self association phenomena were observed [32].

They discovered also significant errors in the structural assignment for currently available preparations of some of these dyes. These errors call into question some

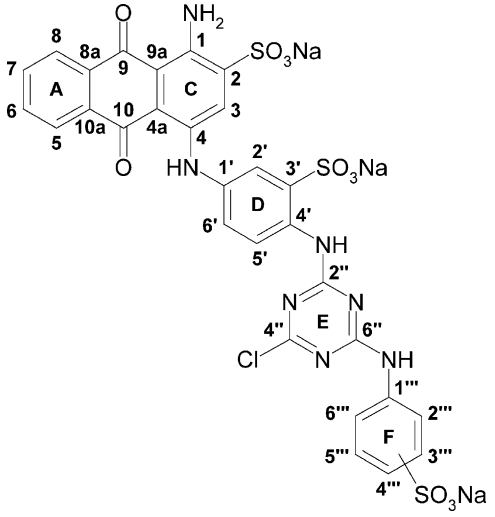

Fig. 1. RB 2, mixture of the terminal ring F *meta* and *para* constitutional isomers.

conclusions made in the past concerning the effect of ligand structures on P2-receptors, since small changes in the structure may cause large differences in the degree of bioselective interaction.

In 1988 Burton et al. synthesised all three terminal ring F isomers of the anthraquinone dye RB 2, again characterised just by TLC and PIRPHPLC. They also analysed a number of commercial dye preparations and in contrast to Hanggi and Carr found them to be composed primarily of the *ortho* sulphoanilino isomer [27].

Burton et al. claimed that Hanggi and Carr seemingly made a false identification of the dichlorotriazinyl analogue of Cibacron blue as the major constituent of those investigated commercial dye preparations [26,27] since they did not investigate the possibility of the third constitutional isomer with the *ortho* sulphonate group.

Additional confusion stems from unfortunate errors in the assignment of generic names, which virtually destroyed the utility of the Colour Index (C.I.) system with respect to these dyes.

The extent of the confusion may be illustrated by generic dye names of the C.I. (3rd Ed. C.I.) [31] for 'RB 2', classified as isomeric *meta*- and *para*-mixture.

Manufacturers dye names		Generic dye names (3rd Ed. C.I.)
Procion Blue HB (ICI)	≅	Procion Blue H-B
Cibacron Blue 3G	≅	Cibacron Blue F-3GA
(Ciba–Geigy)	≅	C.I. RB 2 (C.I. Constitution number 61211)

Nevertheless, Cibacron dyes were predominantly composed of the *ortho*-isomer. Moreover, RB 2 was also sold under the names of Cibacron Blue F3GA and Procion Blue HBS, respectively. All these dyes were withdrawn with the C.I. 3rd Ed. 2nd Revision.

In follow up, a new Cibacron Blue 3GA (CB 3GA) was marketed by BDH, Pierce, and Polysciences, said to contain the *ortho*-isomer different from C.I. RB 2. Sigma re-evaluated their so-called RB 2 material as the F-ring *ortho* isomer, and renamed it as CB 3GA. The ring F *meta* and *para* isomeric mixture was then sold as Basilen Blue E-3G, which was finally replaced by the RBI product RB 2 after the Sigma merger with RBI. Burton et al. advocate either the analysis of commercial preparations of sulphonated dyes prior to biological use or the structure selective synthesis, since unpurified and ill-defined dye samples are hazards of reliable research work including receptor-ligand-binding work [27]. Careful analysis of the purity and structural composition of RB 2 preparations is mandatory but is not guaranteed for most of the published P2-receptor work. Therefore, we have investigated thoroughly the story of the RB 2 chemistry from the beginning.

2. Chemistry

The commercially available dye RB 2 which was used in this study was offered by RBI and distributed by Biotrend (Köln, Germany) in a purity of 97% with respect to the mixture of two constitutional isomers. In comparison, RB 2 products of other suppliers provide merely a dye content of ca. 60%, but have also been used in literature work so far. The same applies to CB 3GA which we purchased from Sigma with a dye content of ca. 65%. In this study CB 3GA was purified by column chromatography from organic impurities as well as from inorganic salts such as NaCl and sodium phosphate. The remaining organic matter was then investigated NMR-spectroscopically as was the purchased RB 2 mixture. RB 2 was found to consist of two constituents in a ratio of 35:65 by intensive ^{1}H- and ^{13}C-NMR investigations. The isomerism was exclusively found in ring F with a *meta*- and *para*-sulfonate group, respectively. These constitutional isomers contained in RB 2 are different from the dye CB 3GA, which is the F-ring-*ortho*-sulfonate isomer of the two RB 2 constituents. The NMR-data of all three anthraquinone derived constitutional isomers are summarised in Table 1 for comparison. Up to now a complete NMR-spectroscopic characterisation was lacking in the literature, due to severe difficulties with the interpretation of the NMR data [32]. Therefore, we purchased and synthesised numerous compounds with comparable structures to RB 2 fragments. Based on the analysed NMR shift values for these compounds we have developed a system of increments which helped us to identify unequivocally the isomers of RB 2 and CB 3GA. 2D-NMR-techniques like ^{1}H,^{1}H-COSY, NOESY, ^{1}H-^{13}C-HETCOR, long-range-^{1}H-^{13}C-, ^{1}H-^{15}N-HETCOR and *J*-resolved spectra were necessary to resolve all the problems in signal identification. Later on we were able to verify the correlation between our NMR data and the corresponding, by now isomerically pure isomers. NMR data obtained from the pure isomers (Table 1) show that chemical shifts for the anthraquinone core and the side chain rings D and E of the two RB 2 isomers differ by less than $\pm$0.2 ppm in ^{13}C- and $\pm$0.04 ppm in ^{1}H-NMR-spectra. The same applies for differences between the CB 3GA *ortho* isomer and the other two isomers, although the differences come up to $\pm$0.7 ppm in ^{13}C- and $\pm$0.1 ppm in ^{1}H-NMR-spectra. These significant chemical shift variations are restricted to terminal ring F

Table 1
^{13}C- and ^{1}H-NMR chemical shifts δ (ppm) of CB 3GA and the constitutional isomers RB 2 *meta* and RB 2 *para*

NMR-signal number	CB 3GA		RB 2 meta		RB 2 para	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	144.2	–	144.2	–	144.2	–
2	142.5	–	142.8	–	142.7	–
3	122.7	8.06	122.5	7.98	122.5	7.97
4	140.7	11.99	141.1	12.05	141.0	12.04
4a	111.6	–	111.1	–	111.2	–
5	125.7	8.26	125.8	8.29	125.8	8.29
6	132.5	7.83	132.4	7.83	132.5	7.85
7	132.5	7.83	132.8	7.83	132.9	7.85
8	125.7	8.26	125.8	8.29	125.8	8.29
8a	134.0	–	134.0	–	134.0	–
9	181.8	–	181.6	–	181.7	–
9a	109.3	–	109.0	–	109.1	–
10	182.4	–	182.2	–	182.3	–
10a	133.4	–	133.5	–	133.5	–
1′	134.0	–	133.3	–	133.5	–
2′	121.7	7.66	121.9	7.57	122.0	7.60
3′	137.4	–	137.1	–	137.2	–
4′	130.7	10.29	131.2	10.32	131.2	10.35
5′	122.1	8.38	121.4	8.5	121.5	8.5
6′	123.5	7.31	124.1	7.33	123.9	7.30
2″	163.4	–	163.9	–	163.7	–
4″	168.3	–	168.2	–	168.3	–
6″	163.2	10.34	162.9	10.26	162.9	10.30
1‴	134.1	–	137.4	–	138.2	–
2‴	136.2	–	118.4	7.83	119.7	7.60
3‴	126.8	7.77	148.7	–	125.8	7.60
4‴	122.6	7.09	121.2	7.75	143.4	7.60
5‴	129.4	7.50	127.7	7.36	125.8	7.60
6‴	121.1	8.30	120.9	7.36	119.7	–

nuclei. This made it difficult to quantify the ratio of the two isomers RB 2 *meta* and RB 2 *para* contained in the RB 2 mixture. In the ^{1}H-NMR spectra, the NH-4′-signal is the only one that features a sufficient shift difference between the two isomers and does not overlap with other signals. In the ^{13}C-spectra, the C-1‴ signals of the two isomers are well separated. In addition, the signals of C-2‴ can be used, considering that the ^{13}C-signal in the *para*-isomer represents the two carbons C-2‴ and C-6‴. Integration of the NH-4′ signal resulted in a ratio of 62:38, integration of the C-1‴ and the C-2‴ signal in a ratio of 66:34 in each case for the benefit of the *para* isomer.

RB 2 as the purified isomeric mixture unfortunately could not be separated by any type of CC into the F-ring *meta* and *para* isomers. Therefore, we synthesised structure-selectively the two constituents contained in RB 2 as shown in Fig. 2.

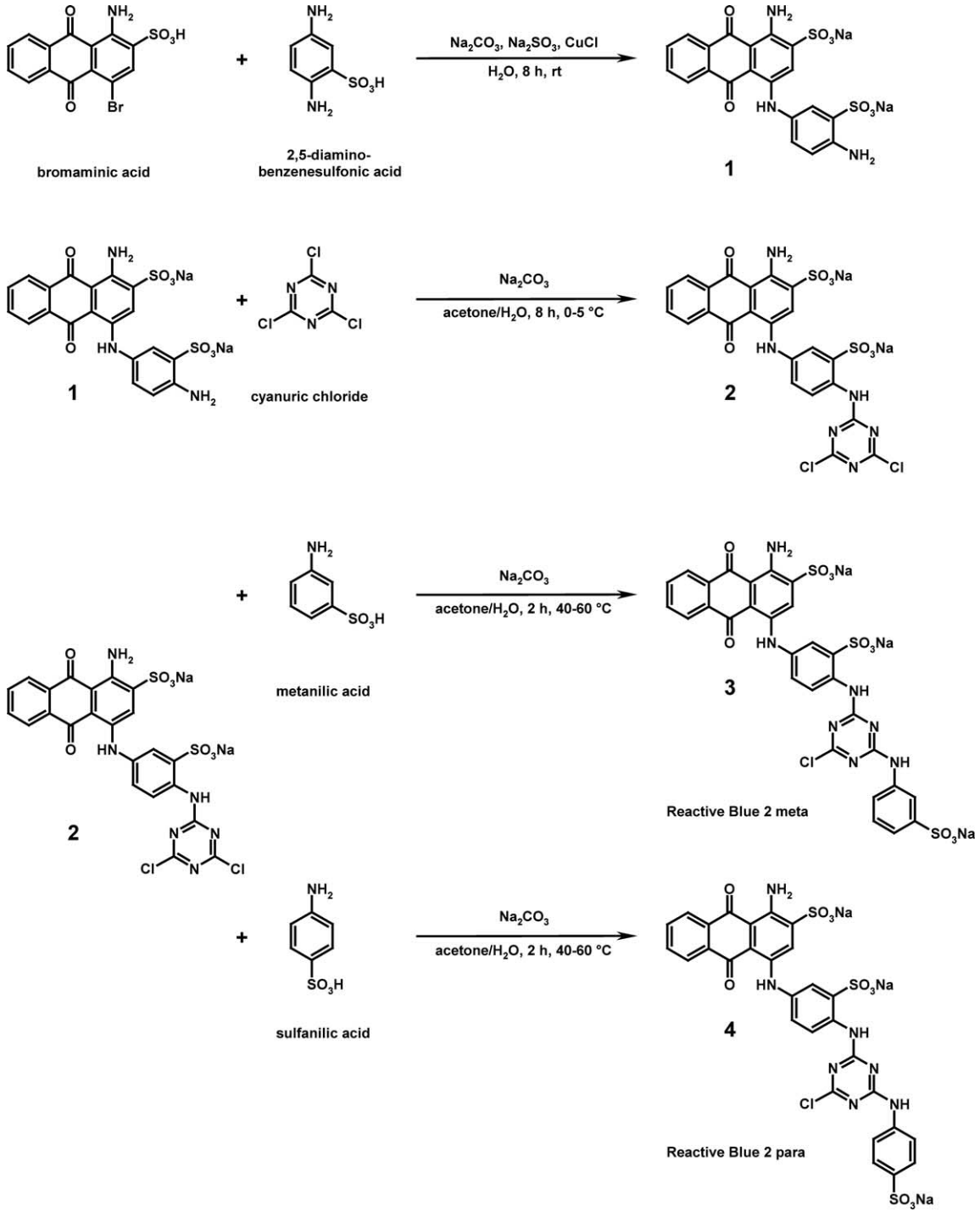

Fig. 2. Synthesis of RB 2 isomers.

The constitutional isomers of RB 2 were synthesised according to procedures of Wojtkiewicz and Kraska [34] and of Hanggi and Carr [26]. In the first step, 1-amino-4-bromo-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (bromaminic acid) (764 mg, 2 mmol) and 2,5-diaminobenzenesulfonic acid (760 mg, 3 mmol) were added to a stirred solution of sodium carbonate (500 mg) and sodium sulphite (400 mg) in water (50 mL) at room temperature (r.t.). Copper(I) chloride (50 mg) was added and the mixture was stirred for ca. 8 h, monitoring the formation of 1-amino-4-(4-amino-3-sulfophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**1**) by reversed phase TLC using a mobile phase of methanol–water (2:3). After completion of the reaction the solution was filtered and the residue was washed with water (3 × 20 mL) and methanol (3 × 20 mL). The combined filtrates were evaporated. The residue was purified by flash column chromatography on RP-18 silica gel using a methanol–water (1:3) eluent. According to our observations a nitrogen atmosphere did not improve the reaction. Higher reaction temperatures as given in the procedure of Pearson et al. [35] did reduce the reaction time, but also the yield of the product on the expenses of a higher amount of side products.

In the next step a stoichiometric amount of cyanuric chloride in an ice-cooled aqueous solution of acetone was added to an aqueous solution of **1** at 0–5 °C. The resulting solution was stirred at 0–5 °C for 1 h while 2 M sodium carbonate solution was added to keep the pH in the range 5–7. Then, in the final reaction step an excess of metanilic and sulphanilic acid, respectively, in an aqueous solution was added to the solution of 1-amino-4-[4-(4,6-dichloro-[1,3,5]triazin-2-ylamino)-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**2**) and the temperature was increased to 40–60 °C. Sodium carbonate was used again to maintain a pH of 5–7. Reaction progress was monitored by analytical reversed phase TLC using again a mobile phase of methanol–water (2:3). Finally the solvent was evaporated and the blue residue was purified by flash column chromatography on RP-18 silica gel using a methanol–water (1:3) eluent to give the constitutional isomers RB 2 *meta* (**3**) and RB 2 *para* (**4**), respectively.

Functional tests were carried out with the RB 2 mixture, the two pure constitutional isomers of RB 2 and with the pure *ortho*-isomer CB 3GA in order to determine the $P2Y_1$-like- versus $P2X_1$-selectivity.

3. Pharmacology

We have previously reported a structure–activity-study [33] in which the purified dyes RB 2, CB 3GA and other structurally related compounds were studied on contractions of the rat vas deferens (RVD) elicited by α,β-methylene ATP (α,β-MeATP; mediated by native $P2X_1$-receptors which are presumably identical to the recombinant $P2X_1$-receptor subtype) and relaxations of the carbachol-precontracted guinea-pig taenia coli (GPTC) elicited by adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS; mediated by native $P2Y_1$-like-receptors which are so far not cloned but pharmacologically similar to the recombinant $P2Y_1$-receptor subtype). In this former study, CB 3GA ($K_d = 1.6$ μM) was considerably more potent than RB 2 ($K_d = 9.9$–11.4 μM) at the $P2X_1$-receptor, whereas CB 3GA ($K_d = 2.9$ μM) and RB 2 ($K_d = 3.4$ μM) were almost equipotent at the $P2Y_1$-like-receptor. It also became clear that RB 2 and CB 3GA display little $P2Y_1$-like- versus $P2X_1$-selectivity (selectivity ratios were 2.9–3.4 and 0.6, respectively). Never-

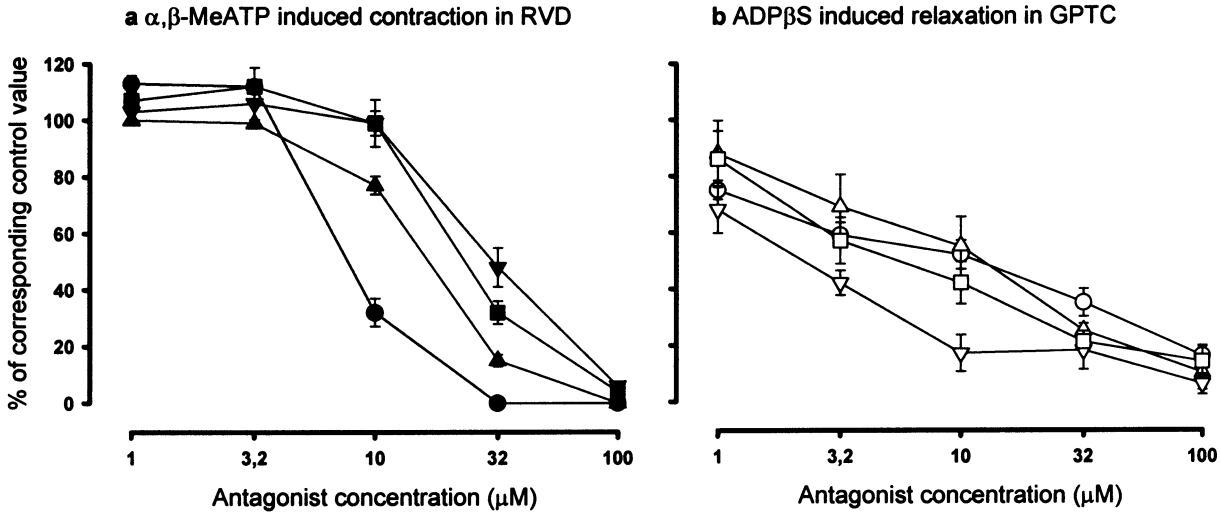

Fig. 3. Effects of antagonists on (a) contraction of RVD elicited by 3.2 μM α,β-MeATP (CB 3GA ●; RB 2 mixture ■; RB 2 meta ▲; RB 2 para ▼; means ± S.E.M. from $n = 5$–11 experiments) and on (b) relaxation of carbachol-precontracted GPTC elicited by 0.1 μM ADPβS (CB 3GA ○; RB 2 mixture □; RB 2 meta △; RB 2 para ▽; means ± S.E.M. from $n = 4$–7 experiments).

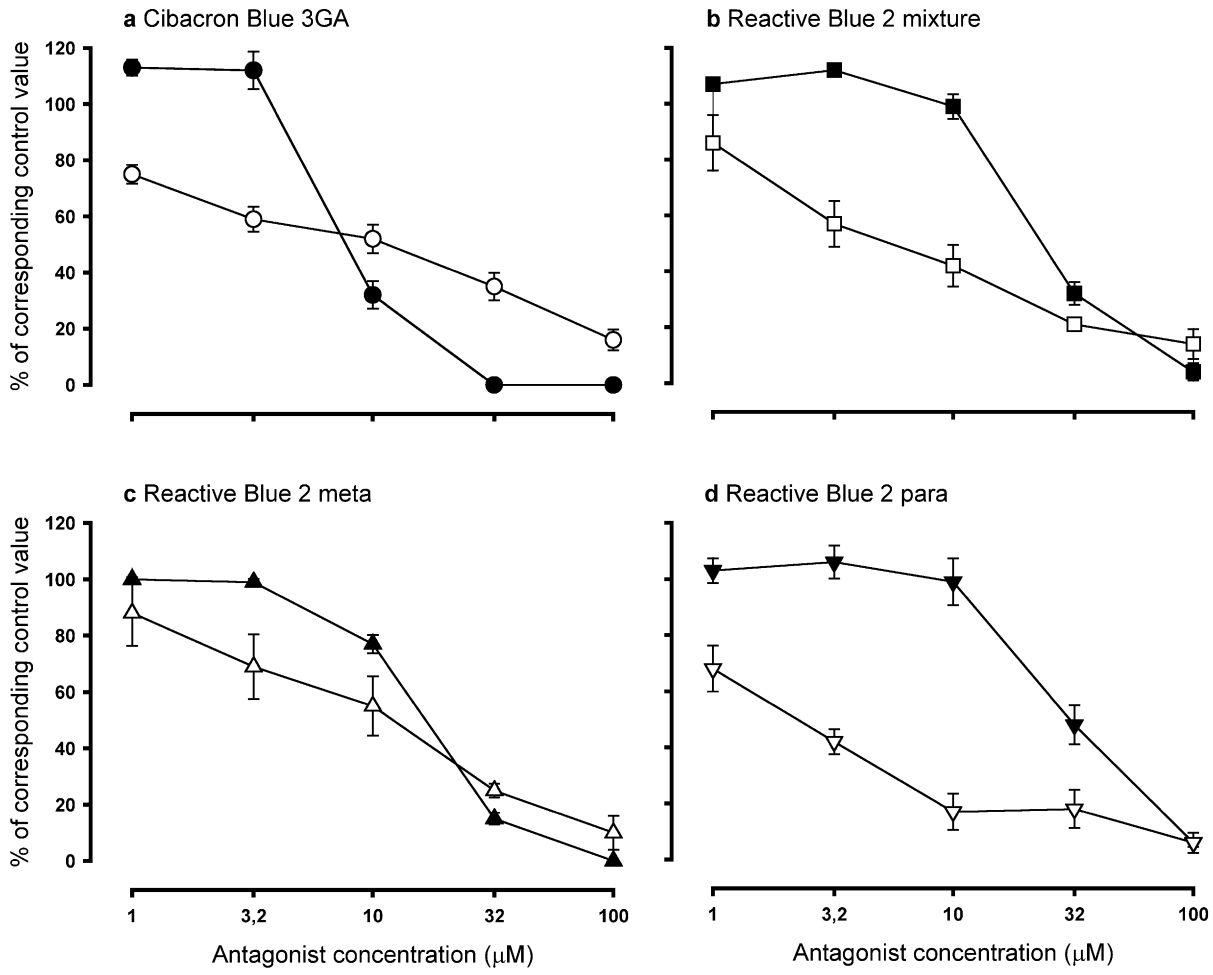

Fig. 4. Selectivity profiles: effects of (a) CB 3GA, (b) RB 2 mixture, (c) RB 2 *meta* and (d) RB 2 *para* on $P2X_1$-receptors of RVD (filled symbols) vs. effects on $P2Y_1$-like-receptors of GPTC (blank symbols). Means ± S.E.M. from $n = 4–11$ experiments.

theless unequivocal and reliable results are only gained from structurally pure entities.

In the present study, purified CB 3GA, the RB 2 mixture and the constitutional isomers of RB 2, RB 2 *meta* and *para*, were investigated to answer the question whether they differ in their inhibitory potencies and selectivities. So we decided to determine the IC_{50} values of all pure constitutional isomers in addition to the RB 2 mixture. Contractions of the RVD were elicited every 60 min by a 3.2 μM concentration of α,β-MeATP. Relaxations of the carbachol-precontracted GPTC were elicited every 60 min by a 0.1 μM concentration of ADPβS. Both agonist concentrations are ca. the EC_{50} values under the present conditions [33,36–39]. The antagonists were added in vivo in increasing concentrations ranging from 1 to 100 μM. IC_{50} values were calculated for each experiment by interpolation from the two nearest data points. Means were tested for a significant difference by the Mann–Whitney test. Concentration response curves of the compounds are shown in Figs. 3 and 4.

The RB 2 mixture and the pure constitutional isomers inhibited the α,β-MeATP induced contraction in RVD, mediated by $P2X_1$-receptors, as well as the ADPβS induced relaxation in GPTC, mediated by $P2Y_1$-like-receptors.

4. Results and discussion

Commercially available CB 3GA and the RB 2 mixture were chromatographically purified and identified by ^1H- and ^{13}C-NMR studies. The two constitutional isomers of RB 2 were synthesised. All compounds were investigated in functional tests.

The results are summarised in Table 2. Comparing the pure constitutional isomers, CB 3GA ($IC_{50} = 9.1$ μM) is the most potent compound at the $P2X_1$-receptor. RB 2 *meta* ($IC_{50} = 19.7$ μM) is more potent than RB 2 *para* ($IC_{50} = 35.5$ μM). At the $P2Y_1$-like-receptor, RB 2 *meta* ($IC_{50} = 12.0$ μM) and CB 3GA ($IC_{50} = 17.4$ μM) are nearly equipotent, whereas RB 2 *para* ($IC_{50} = 2.6$ μM)

Table 2
Antagonist IC_{50} values at $P2X_1$-receptors (RVD), at $P2Y_1$-like-receptors (GPTC), and selectivity ratios $P2Y_1$-like versus $P2X_1$

Compound	IC_{50} (μM)		Selectivity, $P2Y_1$-like vs. $P2X_1$
	$P2X_1$	$P2Y_1$-like	
CB 3GA [a]	9.1±0.8	17.4±4.7	0.5
RB 2 [b]	28.4±2.6	7.7±2.9	3.7
RB 2 *meta*	19.7±1.1	12.0±4.3	1.6
RB 2 *para*	35.5±5.4	2.6±0.4	13.7

IC_{50} values are from experiments of Figs. 3 and 4 ($n = 4–11$). Differences between IC_{50} values at $P2X_1$- and $P2Y_1$-like-receptors are significant for RB 2 and RB 2 *para* ($P < 0.05$).
[a] *Ortho* isomer.
[b] Mixture (35:65) of *meta* and *para* isomers.

reveals stronger inhibition. As a result, the *para*-isomer of RB 2 displays moderate $P2Y_1$-like- versus $P2X_1$-selectivity of 13.7, whereas the other constitutional isomers are non-selective. The commercially available RB 2 mixture with IC_{50}s of 28.4 μM ($P2X_1$) and 7.7 μM ($P2Y_1$-like), respectively, shows a $P2Y_1$-like- versus $P2X_1$-selectivity ratio of 3.7 only.

The results demonstrate that the *meta* RB 2 isomer like the *ortho* isomer CB 3GA is a non selective P2-receptor antagonist whereas the *para*-isomer — contained in RB 2 with 65% only — is a moderate $P2Y_1$-like-selective antagonist.

5. Conclusions

It is clearly demonstrated that RB 2 as a *meta*/*para* mixture does not discriminate the $P2X_1$-receptor of RVD and the $P2Y_1$-like-receptor of GPTC [33] — in contrast to most of published work and to commercial advertisement in receptor ligand catalogues [40] —, whereas the pure *para* isomer of RB 2 is a comparatively potent and selective antagonist at the $P2Y_1$-like-receptor in comparison with the native $P2X_1$-receptor.

The present results prompted us to further investigate other structural modifications of RB 2 and CB 3GA with respect to potency, selectivity and reversibility at the $P2Y_1$-like-receptor. Structure-selectivity-relationships are under exploration and will be published in the near future.

6. Experimental protocols

6.1. Chemistry

6.1.1. Chemicals

Bromaminic acid (1-amino-4-bromoanthraquinone-2-sulphonic acid) was supplied by Bayer AG (Leverkusen, Germany) and purchased from Sigma (Taufkirchen, Germany), 2,5-diaminobenzenesulphonic acid, cyanuric chloride (2,4,6-trichloro-*s*-triazine), *m*- and *p*-aminobenzenesulphonic acids were purchased from Aldrich (Taufkirchen, Germany), CB 3GA from Sigma and RB 2 from RBI (Biotrend, Köln, Germany).

6.1.2. Analytical studies

^{1}H- and ^{13}C-1D- and -2D-NMR spectra were recorded on a Varian Unity 300 spectrometer operating at 299.5 and 75.4 MHz, respectively. Chemical shift values δ (in ppm) are referenced to the signal of DMSO in DMSO-d_6, with $\delta_{TMS} = \delta_{DMSO} - 2.49$ for ^{1}H-NMR and $\delta_{TMS} = \delta_{DMSO} - 39.7$ for ^{13}C-NMR. The chemical shifts of proton multiplets were determined via the corresponding ^{1}H-^{13}C-HETCOR spectra. ^{1}H-NMR data are listed in the following order: multiplicity (s, singlet; d, dublett; t, triplet; m, multiplet), number of nuclei, assignment. Flash chromatography was carried out on Silica gel RP C18 (32–63 μm) (ICN Biomedicals, Eschwege, Germany). TLC was performed on Silica gel aluminium sheets RP-18 F_{254s} (E. Merck, Darmstadt, Germany).

*6.1.2.1. 1-Amino-4-(4-amino-3-sulfophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**1**).* To a stirred solution of 500 mg sodium carbonate and 400 mg sodium sulphite in 50 mL water were added 764 mg (2 mmol) 1-amino-4-bromo-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (bromaminic acid) and 760 mg (4 mmol) 2,5-diaminobenzenesulfonic acid. Then 50 mg copper(I) chloride were added and the mixture was stirred for ca. 8 h, monitoring the formation of product by reversed phase TLC using a mobile phase of methanol–water (2:3). After completion of the reaction in about 8 h the mixture was filtered and the residue was washed with water (3 × 20 mL) and methanol (3 × 20 mL). The combined filtrates were evaporated. The blue residue was separated from impurities by flash column chromatography on reversed phase silica gel using a methanol–water (1:3) eluent. The combined fractions were filtered and evaporated. The blue residue was dried under vacuum.

$C_{20}H_{13}N_3O_8S_2Na_2$ [533.44] ^{1}H-NMR (DMSO-d_6) δ 5.65 (s, 2H, NH_2-4′), 6.70 (d, 1H, H-5′), 6.95 (dd, 1H, H-6′), 7.34 (d, 1H, H-2′), 7.80 (s, 1H, H-3), 7.81 (m, 2H, H-6, H-7), 8.28 (m, 2H, H-5, H-8), 9-11 (br, 2H, NH_2-1), 12.05 (s, 1H, NH-4); ^{13}C-NMR (DMSO-d_6) δ 108.69 (C-9a), 109.47 (C-4a), 116.13 (C-5′), 122.56 (C-3), 124.10 (C-2′), 125.51 (C-1′), 125.67 (C-5), 125.76 (C-8), 126.57 (C-6′), 130.97 (C-3′), 132.29 (C-6), 132.42 (C-7), 133.73 (C-10a), 134.02 (C-8a), 143.04 (C-4), 143.19 (C-4′), 143.58 (C-2), 143.92 (C-1), 181.11 (C-10), 181.34 (C-9).

*6.1.2.2. 1-Amino-4-[4-(4,6-dichloro-[1,3,5]triazin-2-ylamino)-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**2**).* An ice-cooled

solution of 35 mg (0.19 mmol) cyanuric chloride in water (10 mL) and acetone (10 mL) was added to a solution of 100 mg (0.19 mmol) **1** in water (10 mL) at 0–5 °C. The resulting solution was stirred at 0–5 °C for 1 h while 2 M sodium carbonate solution was added dropwise to keep the pH in the range 5–7. The reaction was monitored for completeness by reversed phase TLC using a mobile phase of methanol–water (2:3). After completion of the reaction the solvent was evaporated and the blue residue was purified by flash column chromatography on reversed phase silica gel using a methanol–water (1:3) eluent. The combined fractions were filtered and evaporated. The blue residue was dried under vacuum.

$C_{23}H_{12}N_6O_8Cl_2S_2Na_2$ [681.39] ^{1}H-NMR (DMSO-d_6) δ 7.27 (dd, 1H, H-6′), 7.56 (d, 1H, H-2′), 7.83 (m, 2H, H-6, H-7), 7.98 (s, 1H, H-3), 8.00 (d, 1H, H-5′), 8.27 (m, 2H, H-5, H-8), 10.7 (br, 1H, NH-4′), 9–11 (br, 2H, NH_2-1), 12.0 (br, 1H, NH-4); ^{13}C-NMR (DMSO-d_6) δ 109.19 (C-9a), 111,54 (C-4a), 121.16 (C-2′), 122.61 (C-3), 123.25 (C-6′), 125.63 (C-5′), 125.85 (C-5, C-8), 129.44 (C-4′), 132.58 (C-6), 132.99 (C-7), 133.46 (C-10a), 134.03 (C-8a), 135.00 (C-1′), 139.50 (C-3′), 140.62 (C-4), 142.69 (C-2), 144.27 (C-1), 152.91 (C-2″), 154.27 (C-4″, C-6″), 181.77 (C-9), 182.46 (C-10).

*6.1.2.3. 1-Amino-4-{4-[4-chloro-6-(3-sulfophenylamino)-[1,3,5]triazin-2-ylamino]-3-sulfophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**3**) (RB 2 meta).* A solution of 165 mg (0.95 mmol) of metanilic acid in water (10 mL) was added to the stirred solution of 130 mg (0.19 mmol) **2** in water (20 mL) and acetone (10 mL) at 0–5 °C. Then the temperature was increased to 40–60 °C and stirring was continued for ca. 2 h. A 2 M sodium carbonate solution was added dropwise to keep the pH in the range of pH 5–7. Reaction progress was monitored by reversed phase TLC using a mobile phase of methanol–water (2:3). Finally the solvent was evaporated and the blue residue was purified by flash column chromatography on reversed phase silica gel using a methanol–water (1:3) eluent. The combined fractions were filtered and evaporated. The blue residue was dried under vacuum.

$C_{29}H_{17}N_7O_{11}S_3Na_3Cl$ [840.10] ^{1}H-NMR (DMSO-d_6) δ 7.33 (dd, 1H, H-6′), 7.36 (m, 1H, H-5‴), 7.57 (d, 1H, H-2′), 7.75 (br, 1H, H-4‴), 7.83 (m, 3H, H-2‴, H-6, H-7), 7.98 (s, 1H, H-3), 8.29 (m, 2H, H-5, H-8), 8.5 (br, 1H, H-5′), 7-11 (br, 2H, NH_2-1), 10.26 (s, 1H, NH-6″), 10.32 (s, 1H, NH-4′), 12.05 (s, 1H, NH-4); ^{13}C-NMR (DMSO-d_6) δ 109.04 (C-9a), 111.10 (C-4a), 118.37 (C-2‴), 120.94 (C-6‴), 121.15 (C-4‴), 121.40 (C-5′), 121.85 (C-2′), 122.48 (C-3), 124.07 (C-6′), 125.77 (C-5, C-8), 127.68 (C-5‴), 131.15 (C-4′), 132.43 (C-6), 132.79 (C-7), 133.30 (C-1′), 133.47 (C-10a), 134.00 (C-8a), 137.11 (C-3′), 137.35 (C-1‴), 141.05 (C-4), 142.80 (C-2), 144.20 (C-1), 148.74 (C-3‴), 162.85 (C-2″), 163.88 (C-6″), 168.20 (C-4″), 181,62 (C-9), 182.15 (C-10).

*6.1.2.4. 1-Amino-4-{4-[4-chloro-6-(4-sulfophenylamino)-[1,3,5]triazin-2-ylamino]-3-sulfophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**4**) (RB 2 para).* A solution of 165 mg (0.95 mmol) of sulphanilic acid in water (10 mL) was added to the stirred solution of 130 mg (0.19 mmol) **2** in water (20 mL) and acetone (10 mL) at 0–5 °C. Then the temperature was increased to 40–60 °C and stirring was continued for ca. 2 h. A 2 M sodium carbonate solution was added dropwise to keep the pH in the range of pH 5–7. Reaction progress was monitored by reversed phase TLC using a mobile phase of methanol–water (2:3). Finally the solvent was evaporated and the blue residue was purified by flash column chromatography on reversed phase silica gel using a methanol–water (1:3) eluent. The combined fractions were filtered and evaporated. The blue residue was dried under vacuum.

$C_{29}H_{17}N_7O_{11}S_3Na_3Cl$ [840.10] ^{1}H-NMR (DMSO-d_6) δ 7.30 (dd, 1H, H-6′), 7.60 (m, 5H, H-2′, H-2‴, H-3‴, H-5‴, H-6‴), 7.85 (m, 2H, H-6, H-7), 7.97 (s, 1H, H-3), 8.29 (m, 2H, H-5, H-8), 7-11 (br, 2H, NH_2-1), 10.30 (s, 1H, NH-6″), 10.35 (s, 1H, NH-4′), 12.04 (s, 1H, NH-4); ^{13}C-NMR (DMSO-d_6) δ 109.11 (C-9a), 111.21 (C-4a), 119.68 (C-2‴, C-6‴), 121.50 (C-5′), 121.98 (C-2′), 122.47 (C-3), 123.92 (C-6′), 125.80 (C-3‴, C-5‴), 125.83 (C-5, C-8), 131.19 (C-4′), 132.49 (C-6), 132.86 (C-7), 133.47 (C-1′,C-10a), 134.00 (C-8a), 137.22 (C-3′), 138.24 (C-1‴), 140.99 (C-4), 142.69 (C-2), 143.39 (C-4‴), 144.17 (C-1), 162.89 (C-2″), 163.73 (C-6″), 168.29 (C-4″), 181.69 (C-9), 182.25 (C-10).

6.1.2.5. 1-Amino-4-{4-[4-chloro-6-(2-sulfophenylamino)-[1,3,5]triazin-2-ylamino]-3-sulfophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (CB 3GA). The purchased CB 3GA was purified by flash column chromatography on reversed phase silica gel using a methanol–water (1:3) eluent. The combined fractions were filtered and evaporated. The blue residue was dried under vacuum.

$C_{29}H_{17}N_7O_{11}S_3Na_3Cl$ [840.10] ^{1}H-NMR (DMSO-d_6) δ 7.09 (t, 1H, H-4‴), 7.31 (dd, 1H, H-6′), 7.50 (t, 1H, H-5‴), 7.66 (d, 1H, H-2′), 7.77 (d, 1H, H-3‴), 7.83 (m, 2H, H-6, H-7), 8.06 (s, 1H, H-3), 8.26 (m, 2H, H-5, H-8), 8.30 (d, 1H, H-6‴), 8.38 (d, 1H, H-5′), 7-11 (br, 2H, NH_2-1), 10.29 (s, 1H, NH-4′), 10.34 (s, 1H, NH-6″), 11.99 (s, 1H, NH-4); ^{13}C-NMR (DMSO-d_6) δ 109.30 (C-9a), 111.57 (C-4a), 121.08 (C-6‴), 121.67 (C-2′), 122.05 (C-5′), 122.56 (C-4‴), 122.71 (C-3), 123.52 (C-6′), 125.71 (C-5, C-8), 126.80 (C-3‴), 129.44 (C-5‴), 130.73 (C-4′), 132.48 (C-6, C-7), 132.89 (C-6), 133.39 (C-10a), 133.96 (C-1′, C-8a), 134.09 (C-1‴), 136.17 (C-2‴), 137.38 (C-3′), 140.65 (C-4), 142.45 (C-2), 144.17 (C-1),

163.16 (C-2″), 163.35 (C-6″), 168.32 (C-4″), 181,79 (C-9), 182.42 (C-10).

6.2. Pharmacology

6.2.1. Methods

Male Wistar rats (250–350 g) or guinea pigs of either sex (400–800 g) were decapitated, the vasa deferentia (rat) or the ventral taenia coli (guinea-pig) removed and cleaned of adherent tissue. The incubation medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3, and disodium EDTA 0.03. It was gassed with 95% O_2/5% CO_2 and kept at 37 °C.

6.2.2. Contraction of rat vas deferens

Prostatic thirds of the vas deferens were suspended vertically in a 5.9 mL organ bath. The lower end was fixed and the upper end attached to an isometric force transducer (K30, Hugo Sachs Elektronik, Hugstetten, Germany). The initial tension applied was 9.8 mN (Graphtec thermal pen recorder, Ettlingen, Germany), but tissues subsequently relaxed to ca. 3 mN during a 60 min equilibration period. The medium was replaced every 15 min. Contractions were elicited by α,β-MeATP. α,β-MeATP was washed out immediately after contractions had peaked. α,β-MeATP was added every 60 min at a concentration of 3.2 μM.

6.2.3. Relaxation of guinea-pig taenia coli

Strips of about 10 mm of the taenia coli were suspended vertically in a 5.9 mL organ bath. The lower end was fixed and the upper end attached to a K30 transducer. The resting tension was repeatedly adjusted to 9.8 mN during an initial 60 min equilibration period. The medium was replaced every 15 min. After equilibration, carbachol was added to the medium at 15 min intervals for 2 min each. Initially, the maximal contraction of each strip was determined by a single addition of carbachol (300 nM). During the following two to four applications, the concentration of carbachol giving about 80% of the maximum was determined. This concentration (50–90 nM) was used for the remainder of the experiment, the 15 min rhythm being kept throughout. ADPβS was added during the plateau of the carbachol response and washed out together with the latter after the ensuing relaxation was maximal. Relaxations were expressed as percentage of the respective carbachol contraction. ADPβS was added every 60 min at a concentration of 0.1 μM.

6.2.4. Statistics

IC_{50} values were calculated for each experiment by interpolation from the two nearest data points. Antagonist IC_{50} values are expressed as the arithmetic mean ± S.E.M. from $n = 4$–11 experiments. Means were tested for a significant difference by the Mann–Whitney test. $P < 0.05$ was taken as the limit of statistical significance.

6.2.5. Materials

Adenosine 5′-*O*-(2-thiodiphosphate) trilithium (ADPβS), carbachol chloride and α,β-methylene adenosine 5′-triphosphate dilithium (α,β-MeATP) were purchased from Sigma. All drugs were dissolved in distilled water. Solutions of drugs were added to the organ bath in aliquots not exceeding 100 μL.

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