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ARTICLE *in* DRUG DEVELOPMENT RESEARCH · JANUARY 2003

Impact Factor: 0.77 · DOI: 10.1002/ddr.10145

Published in final edited form as:
Drug Dev Res. 2002 December 1; 57(4): 173–181. doi:10.1002/ddr.10145.

Evidence for the Recognition of Non-Nucleotide Antagonists Within the Transmembrane Domains of the Human $P2Y_1$ Receptor

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Abstract

Site-directed mutagenesis was used to search for amino acid residues of the human $P2Y_1$ receptor involved in the binding of the P2 receptor antagonists pyridoxal-5′-phosphate-6-azophenyl-2,4-disulfonate (PPADS), its analogue 6-(2′-chloro-phenylazo)-pyridoxal-α^5-phosphate (MRS 2210), the suramin analogue 8-8′-[carbonylbis(imino-3,1-phenylene)]bis(1,3,5-naphthalene-trisulfonate) (NF023), and Reactive blue 2. Receptors containing single amino acid replacements at positions in transmembrane helical domains (TMs) 3, 5, 6, and 7 critical for the activation of the receptor by nucleotide agonists were expressed in COS-7 (African green monkey kidney) cells. Inositol phosphate accumulation was induced by 2-methylthioadenosine 5′-diphosphate (2-MeSADP). In wild type human $P2Y_1$ receptors, PPADS (10 to 60 μM), MRS 2210 (10 μM), NF023 (100 μM), and Reactive blue 2 (10 μM) shifted the concentration-response curve of 2-MeSADP in a parallel manner to the right. For PPADS, a pA_2 value of 5.2 was estimated. The shifts caused by MRS 2210, NF023, and Reactive blue 2 corresponded to apparent pK_B values of 5.6, 5.0, and 5.8, respectively. In K280A mutant receptors, the affinities of PPADS, MRS 2210, NF023, and Reactive blue 2 were about 6- to 60-fold lower than those observed at wild type receptors. The K280A mutation also caused an approximately 1,000-fold increase in the EC_{50} value of the agonist 2-MeSADP, similar to previous observations. In contrast, no major change in antagonistic potency was observed at receptors with other mutations in TMs 3, 5, 6, and 7. Thus, the residue Lys^{280} (6.55), which is located within the upper third of TM 6 of the human $P2Y_1$ receptor, is not only critical for the activation of the receptor but also plays an important role in the binding of pyridoxal derivatives and a number of other chemically unrelated P2 receptor antagonists. Lys^{280} seems to belong to an overlapping region of the respective binding sites.

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Strategy, Management and Health Policy

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Keywords

antagonists; G protein-coupled receptors; mutagenesis; phospholipase C; nucleotides

INTRODUCTION

Extracellular nucleotides act in cellular signalling through two families of membrane-bound receptors: P2Y-subtypes, G protein-coupled receptors (GPCRs) activated by both adenine and uracil nucleotides; and P2X-subtypes, ligand gated ion channels activated by adenine nucleotides [Fredholm et al., 1994; Ralevic and Burnstock, 1998; Khakh et al., 2001; Jacobson et al., 2002]. At least seven P2X- and seven P2Y-subtypes have been cloned in mammalian species. The $P2Y_1$ receptor subtype is a phospholipase C-activating receptor [Harden et al., 1988] present in blood platelets, heart, skeletal muscle, various smooth muscles, and neuronal tissues. Mice in which the $P2Y_1$ receptor gene has been disrupted show impaired platelet aggregation and a reduced incidence of lethal thrombosis [Fabre et al., 1999; Léon et al., 1999], suggesting that a selective antagonist may be useful in antithrombotic therapeutics. At this receptor, the potency order for activation is 2-methylthioadenosine 5′-diphosphate (2-MeSADP)>ADP>2-methylthioadenosine 5′-triphosphate >ATP, while AMP and UTP are inactive [Palmer et al., 1998].

In the absence of physically determined 3D structures for the rhodopsin-like GPCRs, numerous ligand binding sites, including those for biogenic amines and adenosine within the transmembrane helical domains (TMs), have been probed extensively using site-directed mutagenesis [van Rhee and Jacobson, 1996]. Agonist action at both $P2Y_1$ [Jiang et al., 1997; Moro et al., 1998] and $P2Y_2$ receptors [Erb et al., 1995] is dependent on specific residues within TMs 3, 5, 6, or 7. A cluster of positively charged amino acids, Lys and Arg residues near the exofacial side of these TMs, putatively co-ordinates the phosphate moieties of nucleotide ligands. The binding of both nucleotide agonists and a structurally related antagonist, 2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate (MRS 2179) [Camaioni et al., 1998], have previously been modelled using a rhodopsin template, thus confirming the consistency of this hypothesis with energetically favourable conformations of the receptor [Moro et al., 1998].

Few competitive antagonists are known for P2 receptors [Boyer et al., 1994; Jacobson et al., 1999; Lambrecht, 2000], among them the non subtype-selective pyridoxal-5′-phosphate-6-azophenyl-2,4-disulfonate (PPADS) [Lambrecht et al., 1992; Lambrecht, 2000]. Since this antagonist may be considered as a structural lead for the design of novel antagonists, we have now characterised the determinants of recognition of PPADS in human $P2Y_1$ receptors. The identification of the amino acid residues possibly involved in the binding of agonists and antagonists at the $P2Y_1$ receptor may also be important for the general understanding of the molecular mechanisms of the activation process of a GPCR. In some experiments, effects of three other P2 receptor antagonists, namely Reactive blue 2 [see Boyer et al., 1994], the PPADS analogue 6-(2′-chloro-phenylazo)-pyridoxal-α^5-phosphate [MRS 2210; Kim et al., 1998], and the suramin analogue 8-8′-[carbonylbis(imino-3,1-phenylene)]bis (1,3,5-naphthalene-trisulfonate) [NF023; Bültmann et al., 1996; Lambrecht, 1996, 2000] were studied in addition (for chemical structures see Fig. 1). The results have been presented in part in abstract form [Guo et al., 1999].

METHODS

Materials

The compounds used were: 2-MeSADP trisodium, pyridoxal-5′-phosphate-6-azophenyl-2,4-disulfonate (PPADS) tetrasodium, Reactive blue 2 (Sigma-RBI, St. Louis, MO); 8-8′-[carbonylbis(imino-3,1-phenylene)]-bis(1,3,5-naphthalene-trisulfonate) (NF023) sodium (Calbiochem, San Diego, CA); and 6-(2′-chlorophenylazo)-pyridoxal-α^5-phosphate (MRS 2210; synthesised in our laboratory) [compound 12 in Kim et al., 1998].

Molecular Biology

The preparation of plasmids containing the coding sequence for the human $P2Y_1$ receptor or mutant receptors within the pCD-PS expression vector has previously been described [Jiang et al., 1997]. All constructs contained the influenza virus hemagglutinin-epitope tag sequence at the N-terminus and a hexa-His tag at the C-terminus. The influenza virus hemagglutinin-epitope tag has previously been used to demonstrate the expression of the constructs in the plasma membrane of COS-7 (Chinese hamster ovary) cells [Jiang et al., 1997].

COS-7 (African green monkey kidney) cells were transfected with the receptor plasmids (8 μg DNA per 150 mm dish) approximately 24 h after seeding of the cells (about 4×10^6 cells per dish) using the diethylaminoethyl-dextran method. The transfected cells were grown for an additional 36 h at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Gaithersburg, MD) supplemented with 10% foetal bovine serum (Life Technologies), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM glutamine (Life Technologies).

Pharmacology

Inositol phosphate determination was used as the measure of receptor-stimulated phospholipase C activity. Approximately 24 h before the assay, the culture medium was replaced by DMEM containing no inositol (Life Technologies, cat. no. 11968-021) and no serum but [^{3}H]myo-inositol (final specific activity 2 μCi ml^{-1}; American Radiolabeled Chemicals, St. Louis, MO). The assay was performed at 37°C after preincubation of the cells with 10 mM LiCl for 20 min [Harden et al., 1988; Jiang et al., 1997]. The agonist was applied for 30 min. Antagonists were added either 5 or 20 min before the agonist. Total [^{3}H]inositol phosphates were separated by column chromatography. The tritium content of the samples was determined by liquid scintillation counting.

Half-maximal concentrations (EC_{50} values) for the agonist 2-MeSADP at wild type and mutant receptors in the absence and presence of antagonists were determined by fitting the sigmoid-shaped function "effect=$(E_{max} \times [\text{agonist}]^{slope})/([\text{agonist}]^{slope}+EC_{50}^{slope})$" [Waud, 1976] to concentration-response data obtained in a single experiment. Apparent affinity constants for antagonists, i.e., K_B or pK_B ($-\log_{10} K_B$) values, were calculated from the increases in EC_{50} values in the presence of an antagonist according to the equation "$pK_B = \log_{10}(\text{dose ratio} - 1) - \log_{10}[\text{antagonist}]$" [Furchgott, 1972]. Since data from experiments with only one antagonist concentration were used for this calculation (and, hence, a competitive character of the interaction has not been proven), the calculated values are *apparent* affinity constants. pA_2 values were determined by linear regression of data obtained in experiments with three concentrations of the antagonist similarly as described by Arunlakshana and Schild [1959].

Where appropriate, means±S.E.M. of *n* observations are given. Differences between means were tested for significance by the Student's *t*-test or (multiple comparisons) the one-way

analysis of variance followed by the Student's *t*-test modified according to Bonferroni. $P < 0.05$ or lower was the significance criterion.

RESULTS

Wild type and mutant $P2Y_1$ receptors were transiently expressed in COS-7 cells. Agonist-promoted activation of phospholipase C and its antagonism were measured to determine the ligand recognition properties of the mutant receptors. After preincubation with [^{3}H]myo-inositol, the cells were stimulated by addition of the agonist 2-MeSADP for 30 min as described previously [Jiang et al., 1997]. In cells expressing a receptor construct, 2-MeSADP increased the accumulation of inositol phosphates in a concentration-dependent manner (Fig. 2), while control cells had almost no response to 2-MeSADP. Table 1 shows the maximal increases in [^{3}H]inositol phosphate turnover in dpm and the respective EC_{50} values for the agonist 2-MeSADP at wild type (1.2±0.2 nM, $n = 3$) and mutant human $P2Y_1$ receptors. At the H132A, H277A, K280A, Q307A, and R310K mutant receptors, the potency of the agonist was greatly diminished, similar to the previous results [Jiang et al., 1997], although the maximal increases in [^{3}H]inositol phosphate turnover at the highest concentration of the agonist examined (up to 100 μM) were similar to or even higher than in wild type receptors. Figure 2 shows the respective concentration-response curves for wild type receptors and F131A, F226A, K280A, and Q307A mutants.

In a first series of experiments, the P2 receptor antagonist PPADS or its solvent (control) was added 5 min before the agonist 2-MeSADP. At the wild type human $P2Y_1$ receptor, PPADS added at increasing concentrations of 20, 40, and 60 μM caused increasing as well as parallel shifts of the concentration-response curve of 2-MeSADP to the right (Fig. 2a) without any decrease in the maximal responses (see Table 1) or a significant change in the basal accumulation of radioactivity (see legend to Table 1). The shift caused by PPADS (40 μM) corresponded to an apparent pK_B ($-\log K_B$) value of 5.0 (first line of Table 1).

At F131A, H132A, F226A, H277A, K280R, Q307A, and R310K mutant receptors, the PPADS-induced shifts of the concentration-response curves to the right were similar to those observed at wild type receptors (Fig. 2 and Table 1). In contrast, at K280A mutant receptors there were no or only very small shifts of the concentration-response curves to the right (Fig. 2d and Table 1), demonstrating an obvious loss in antagonistic potency of PPADS. The comparison of the apparent affinity constants for PPADS (estimated from the experiments with PPADS 40 μM) indicates a 6.7-fold reduction in the affinity at the K280A mutant receptor (Table 1). PPADS did not decrease the maximal responses to 2-MeSADP (Table 1) or the basal accumulation of radioactivity (not shown) at any of the mutant receptors studied.

P2 receptor antagonists are known to require a relatively long time for achieving their maximal effects [Leff et al., 1990]. An insufficient preincubation period may cause an underestimation of the antagonistic potency. Therefore, PPADS was applied 20 min before the addition of the agonist 2-MeSADP in a second series of experiments. In wild type receptors, PPADS again shifted the concentration-response curve of 2-MeSADP to the right (Fig. 3a) without any decrease in the maximal responses and with a reduction by about 30% maximally in the basal accumulation of radioactivity (not shown). An analysis according to Arunlakshana and Schild [1959] revealed a slope of 0.95 (not different from unity). Refitting the data with a slope fixed to unity yielded a pA_2 value of PPADS of 5.23 at the wild type $P2Y_1$ receptor (Fig. 4). At K280A mutant receptors, PPADS again caused only small shifts of the concentration-response curves (Fig. 3b). There were no changes in the maximal responses or in the basal accumulation of radioactivity in the presence of PPADS (not shown). The Schild-plot analysis illustrates the loss of the antagonistic potency of PPADS at

this mutant receptor (Fig. 4). The differences in the x-axis intercepts of the Schild-plot regression lines correspond to a 6.2-fold reduction of the affinity of PPADS at the K280A mutant receptor.

In a final series of experiments, the effects of three other P2 receptor antagonists were compared at wild type and K280A mutant receptors. MRS 2210 is an analogue of PPADS, whereas NF023 and Reactive blue 2 are chemically unrelated (see Fig. 1). The antagonists were applied 20 min before the addition of the agonist 2-MeSADP. At wild type receptors, the antagonists caused clear shifts of the concentration-response curve to the right (Fig. 5a). The corresponding apparent pK_B values amounted to 5.6 for MRS 2210, 5.0 for NF023 and 5.8 for Reactive blue 2, respectively, at the human $P2Y_1$ receptor (Table 2). At K280A mutant receptors, MRS 2210, NF023 as well as Reactive blue 2 caused almost no or a very small shift in the concentration response curves (Fig. 5b and Table 2). The change in the estimated affinity constants indicates that the affinity of Reactive blue 2 is about 7-fold lower at the K280A mutant receptor than at the wild type receptor (Table 2). The loss in affinity at the K280A mutant may be even higher for MRS 2210 and NF023 (18- and 67-fold, respectively; however, in these cases the calculated estimates for the apparent K_B values at the mutant are much higher than the applied concentrations). At wild type but not at K280A mutant receptors, Reactive blue 2 and NF023 reduced the basal accumulation of radioactivity by 22 and 25%, respectively (not shown). There were no changes in the maximal responses.

DISCUSSION

Site-directed mutagenesis was used to search for amino acid residues involved in the binding of non-nucleotide antagonists at the human $P2Y_1$ receptor. To this end, the potencies of several P2 receptor antagonists were determined in functional interaction experiments at wild type and mutant receptors. While site-direct mutagenesis experiments allow limited conclusions about the involvement of amino acid residues in the binding of *agonists* (since the mutation could affect both the binding of the agonist to the receptor and the ability of the agonist to activate the receptor) [for discussion see Colquhoun, 1998], a loss in *antagonistic* potency due to a replacement of an amino acid provides more direct evidence for an involvement of this residue in the binding of the ligand. However, it cannot be excluded that an alteration in the topography of the receptor protein influences the results. Therefore, changes in antagonist potencies argue in favour of an involvement of the respective amino acid in the binding of the ligand but cannot directly prove this assumption.

COS-7 cells were transiently transfected with plasmids encoding for the human $P2Y_1$ receptor or mutant receptors. The wild type and the mutant receptors have previously been shown to be accessible to the extracellular medium by virtue of the influenza virus hemagglutinin protein-tag, as detected in an enzyme-linked immunosorbent assay [Jiang et al., 1997]. The agonist 2-MeSADP increased the accumulation of inositol phosphates in a concentration-dependent manner. The variability in the EC_{50} values for the agonist between the different series of experiments was most likely due to a different rate of receptor expression (not further analysed in the present study). However, since experiments in the absence and presence of antagonists for one series were always performed in parallel using the same batch of cells, the determination of antagonist affinities is unlikely to be affected by these changes.

At the human wild type $P2Y_1$ receptor, the antagonists PPADS, MRS 2210, NF023, and Reactive blue 2 shifted the concentration-response curve of 2-MeSADP to the right with pA_2 or apparent pK_B values of 5.2, 5.6, 5.0, and 5.8, respectively (antagonist preincubation period of 20 min). MRS 2210 was selected for this study since it was more potent than

PPADS at the turkey erythrocyte $P2Y_1$ receptor [Kim et al., 1998]. The present results indicate that also at the human $P2Y_1$ receptor, MRS 2210 displays slightly enhanced potency, in spite of the lack of aryl sulfonate groups. MRS 2210 is also less highly charged than PPADS, and thus may serve as a suitable lead compound for the synthesis of new antagonists. Furthermore, it is inactive at the guinea pig urinary bladder P2X receptor and, therefore, may be more selective for P2Y vs. P2X receptors. The pA_2 value of 5.2 of PPADS, estimated in the present study for its affinity at the human $P2Y_1$ receptor, is similar to an apparent pK_B value of about 5.5 calculated from data obtained at the cloned and expressed rat $P2Y_1$ receptor [fig. 1 of Schachter et al., 1997]. It is also similar to the pA_2 value of 5.46 determined at the endogenous $P2Y_1$ receptor in the rat mesenteric arterial bed [Lambrecht, 1996]. At the cloned and expressed turkey $P2Y_1$ receptor, a higher pA_2 value of 5.98 was estimated for PPADS; however, in these experiments the slope of the Schild plot regression line differed from unity [Charlton et al., 1996]. It should be noted that we did not prove the competitive character of the interaction of MRS 2210, NF023, and Reactive blue 2 with the human $P2Y_1$ receptor; but the present results do also not argue against a competitive antagonism. The apparent pK_B value of 5.0 found in the present study as an estimate for the affinity of NF023 at the human $P2Y_1$ receptor agrees with the pA_2 value of 4.94 at the endogenous $P2Y_1$ receptor in the rat mesenteric arterial bed [Lambrecht, 1996].

The present study demonstrates that the replacement of Lys at the position 280 in TM6 by Ala diminished the potency of the four antagonists tested, namely the pyridoxal phosphate analogues PPADS and MRS 2210 as well as the structurally unrelated compounds Reactive blue 2 and the suramin analogue NF023. This argues in favour of an involvement of Lys^{280} in the binding of all four antagonists. Lys^{280} (6.55) corresponds to an Asn residue in the human A_3 adenosine receptor recently found to be essential for the recognition of both agonist and antagonist and proposed to form a H-bond with the exocyclic NH [Gao et al., 2002]. In contrast to Lys^{280}, the residues Phe^{131} and His^{132} in TM3, Phe^{226} in TM5, His^{277} in TM6 as well as Gln^{307} and Arg^{310} in TM7 apparently play no or no major role in the binding of PPADS. Since the replacement of Lys^{280} by Arg did not change the antagonistic potency of PPADS, the positive charge of the residue may be important for the binding of the negatively charged antagonist. In contrast to PPADS (as well as to Reactive blue 2 and NF023), MRS 2210 contains no sulfonate group. Nevertheless, the replacement of Lys^{280} by Ala markedly diminished the antagonistic potencies of both PPADS and MRS 2210. It seems, therefore, possible to speculate that the 5′-phosphate group, identical in PPADS and MRS 2210, but not the sulfonate groups present only in PPADS, interacts with the residue Lys^{280}.

At all mutants tested in the present study, the EC_{50} values for the agonist 2-MeSADP were increased, compatible with a role of these amino acid residues in the agonist binding or the activation process of the receptor as already proposed by Jiang et al. [1997]. Similarly, in a site-directed mutagenesis study on the murine $P2Y_2$ receptor His^{262} and Arg^{265} in TM 6 as well as Arg^{292} in TM 7 (corresponding to His^{277}, Lys^{280}, and Gln^{307} of the human $P2Y_1$ receptor) have been shown to be important for the receptor activity [Erb et al., 1995]. A special role of Phe^{226}, Lys^{280}, and Gln^{307} of the human $P2Y_1$ receptor in the binding of nucleotide derivatives was proven by a marked decrease in the antagonistic potency of the nucleotide antagonist MRS 2179 after a replacement of the residues by Ala [Moro et al., 1998]. In contrast, only the replacement of Lys^{280} by Ala clearly decreased the antagonistic potency of PPADS (present study). These differences may indicate that the binding sites for nucleotide ligands and non-nucleotide P2 antagonists at the $P2Y_1$ receptor are not identical. Nevertheless, there seems to be an overlapping region of binding near residue Lys^{280}. This view is compatible with the notion that PPADS acts as a competitive antagonist at the $P2Y_1$ receptor assuming competition with nucleotides at a common binding site. While our results provide experimental evidence for an interaction of PPADS and related antagonists with the

$P2Y_1$ receptor protein, additional effects of the antagonist downstream from the receptor are not excluded [see Shehnaz et al., 2000].

In conclusion, the present findings indicate that the binding sites for nucleotide ligands and non-nucleotide P2 antagonists at the $P2Y_1$ receptor are overlapping, but not identical. There seems to be a shared area in the region of residue Lys^{280}, which is located in the upper third of TM 6 of the receptor protein. Hence, the results suggest that competition at the binding site plays a role in the antagonistic action. An interesting question is whether this portion of the receptor is also involved in the agonist-induced activation process of the receptor protein.

Acknowledgments

Ivar von Kügelgen is grateful to the Deutsche Forschungsgemeinschaft (Bonn, Germany), for postdoctoral fellowship support. We thank Dr. Carsten Hoffmann (NIDDK/NIH, Bethesda, MD) and Drs. T. Kendall Harden and Robert A. Nicholas (University of North Carolina, Chapel Hill, NC) for helpful discussions.

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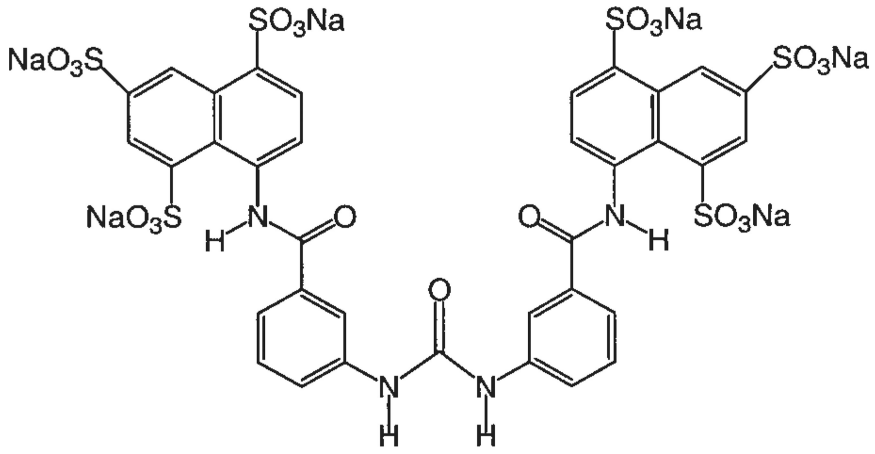

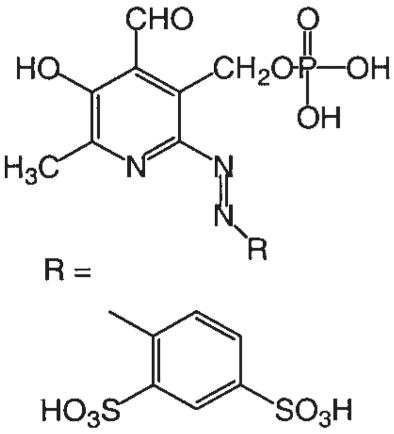

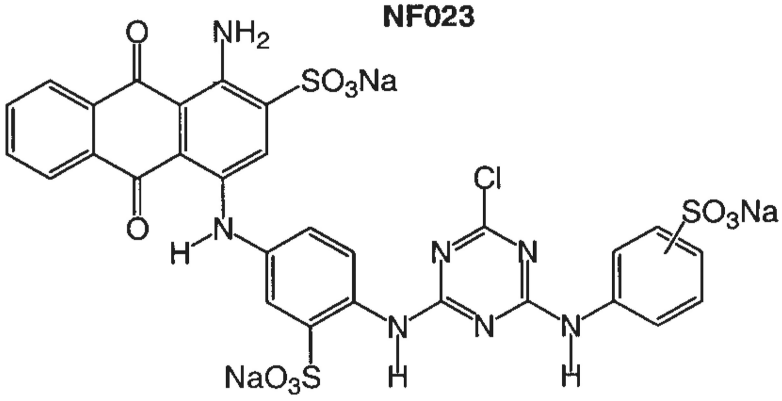

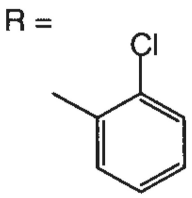

Fig. 1.
Chemical structures of the antagonists used.

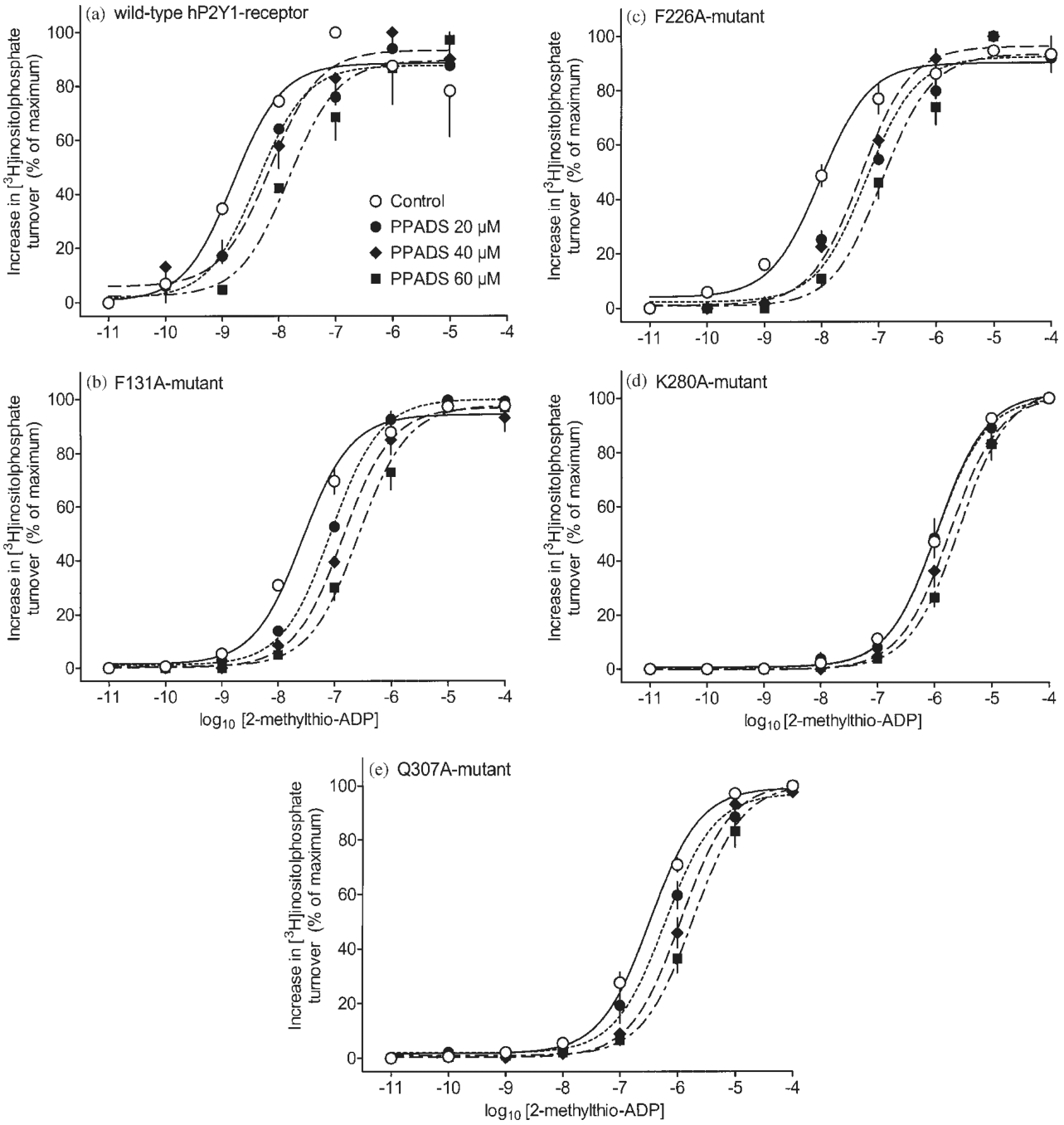

Fig. 2.

2-Methylthioadenosine 5′-diphosphate (2-MeSADP)-induced increases in [^{3}H]inositol phosphate turnover in COS-7 cells transfected with wild type and mutant human $P2Y_1$ receptors (see legend to Table 1) as well as interaction with the P2 receptor antagonist PPADS. 2-MeSADP was applied for 30 min at 37°C. PPADS or its solvent (control) was added 5 min before 2-MeSADP. The agonist-induced increases in inositol phosphate turnover were expressed as percentage of maximum (maximal response in each experiment). A sigmoid-shaped function was fitted to the data. Means±S.E.M. of 3 to 5 experiments. Absolute maxima as well as calculated EC_{50} values are given in Table 1.

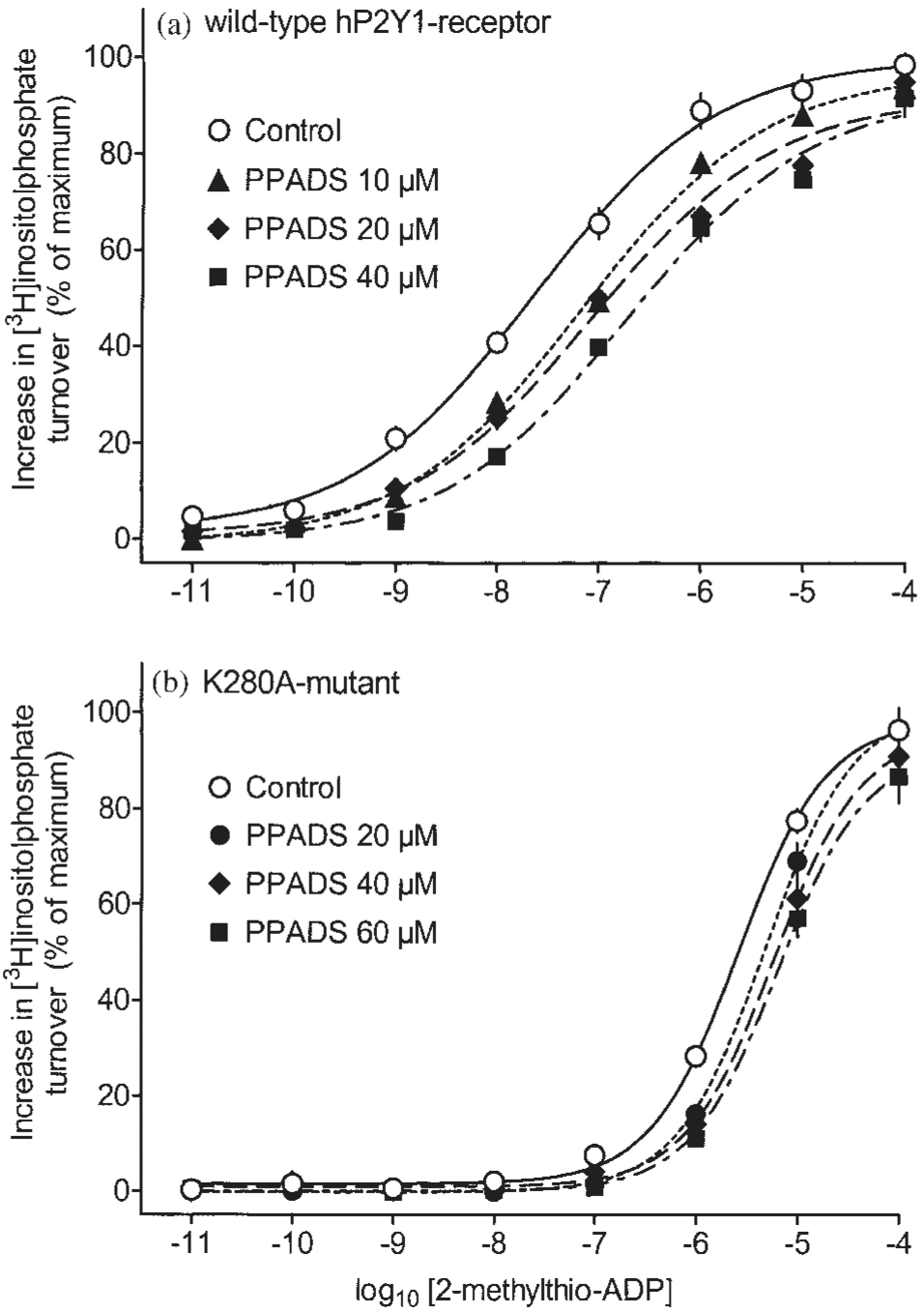

Fig. 3.

Antagonistic effect of PPADS at wild type (**a**) and K280A mutant (**b**) $P2Y_1$ receptors after a longer preincubation period with PPADS of 20 min. PPADS or its solvent (control) was added 20 min before the agonist 2-MeSADP. For further details see legend to Figure 2. Means±S.E.M. of 3 to 6 experiments.

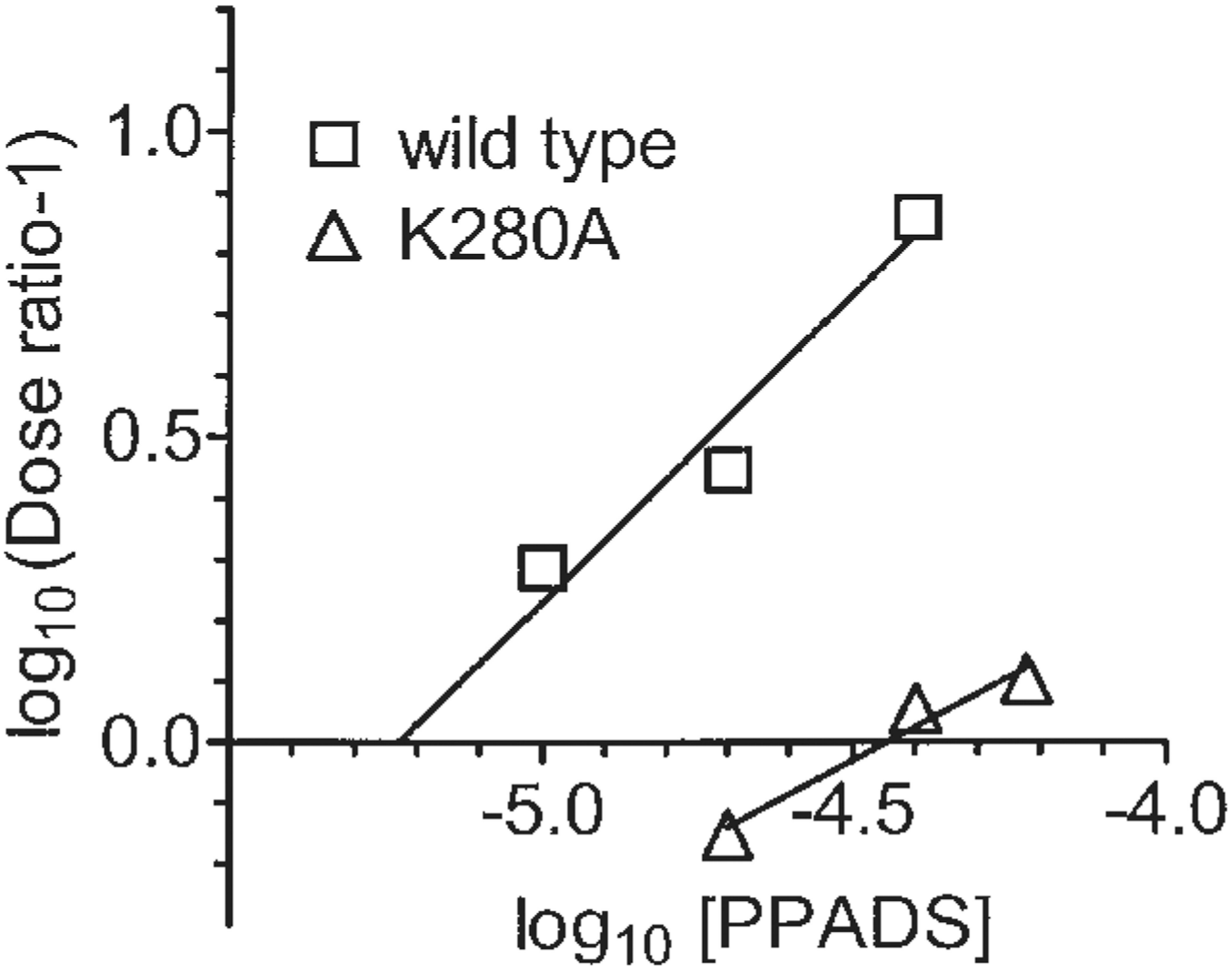

Fig. 4.

Schild plot analysis of interaction experiments with 2-MeSADP and PPADS at wild type and K280A mutant human $P2Y_1$ receptors shown in Figure 3. PPADS was added 20 min before the agonist. Dose ratios were calculated from the increases in averaged EC_{50} values. The slopes of the regression lines amounted to 0.95 (wild type receptor; not different from unity) and 0.55 (K280A mutant; significant different from unity). Since the slope did not significantly differ from unity in experiments at wild type receptors, the respective data were refitted with a slope fixed to unity. The intercepts of the lines at the x-axis amounted to −5.23 (wild type receptor) and −4.44 (K280A mutant).

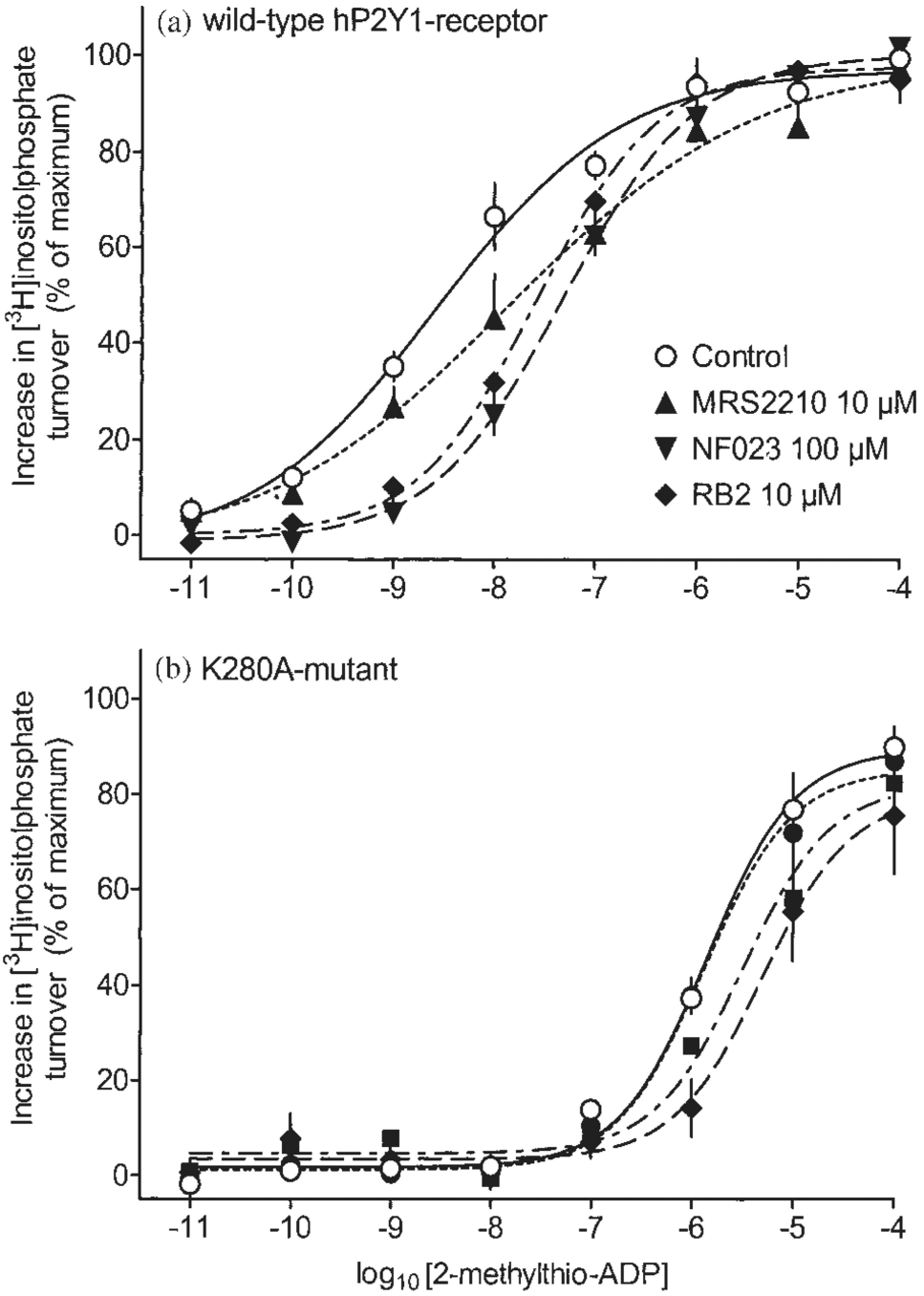

Fig. 5.

Antagonistic effects of MRS 2210, NF023, and Reactive blue 2 at wild type (**a**) and K280A mutant (**b**) $P2Y_1$ receptors. MRS 2210 (10 µM), NF023 (100 µM), Reactive blue 2 (10 µM), or their solvent (control) were added 20 min before 2-MeSADP. For further details, see legend to Figure 2. Means±S.E.M. of 3 to 6 experiments. Calculated EC_{50} values are given in Table 2.

TABLE 1

2-MeSADP-Induced Increases in [^{3}H]Inositol Phosphate Turnover in COS-7 Cells Transfected With Wild Type and Mutant Human P2Y$_1$Receptors and Interaction With the P2 Receptor Antagonist PPADS[a]

Construct	Control			PPADS 40 μM			
	Maximum (2-MeSADP) [dpm]	EC_{50} (2-MeSADP) [nM]	*n*	Maximum (2-MeSADP) [dpm]	EC_{50} (2-MeSADP) [nM]	K_B (PPADS) [μM]	*n*
Wild type	192,000±69,000	1.2±0.2	3	203,000±38,000	6.3±0.5 *	9.0±0.3	3
F131A	168,000±40,000	27.3±5.9	5	157,000±31,000	149±34 *	10.1±4.6	3
H132A	170,000±6,900	186±17	4	152,000±2,300	1,270±40 *	6.7±1.0	3
F226A	118,000±10,300	9.2±2.9	5	98,000±11,500	42.6±8.7 *	8.6±2.3	3
H277A	531,000±39,000 ***	541±98 ***	4	497,000±30,000 ***	3,530±1110 *, ***	8.6±2.0	4
K280A	309,000±33,000	1,160±130 ***	3	276,000±57,000	2,090±390 **	60.5±15.1 ***	3
K280R	307,000±79,000	58.2±21.8	4	279,000±64,000	371±60 *	7.5±2.8	4
Q307A	214,000±49,000	325±37 **	4	140,000±49,000	1,270±360 *	22.0±8.3	4
R310K	803,000±16,000 ***	818±70 ***	4	811,000±28,000 ***	3,560±450 *, ***	13.5±3.9	3

[a]2-MeSADP was applied at increasing concentrations for 30 min at 37°C. PPADS or its solvent (control) was added 5 min before 2-MeSADP. Maximum: maximal increases in [^{3}H]inositol phosphate turnover induced by 2-MeSADP (in decays per min, dpm). The basal accumulation of radioactivity in cells transfected with the wild type receptor amounted to 13,600±6,500 dpm (n = 3) in the absence of PPADS and 25,400±5,000 dpm (n = 3) in the presence of PPADS 40 μM. Half-maximal concentrations (EC_{50} values) for 2-MeSADP were determined by fitting a sigmoid-shaped function to the data of each experiment. Experiments in the absence or presence of PPADS were performed in parallel. Apparent affinity constants (K_B) values for PPADS were calculated from the respective increases in EC_{50} values. Means±S.E.M. of (n) experiments.

*Statistically significant differences versus respective value in the absence of PPADS ($P < 0.05$; t-test).

** and ***Statistically significant differences vs. respective value in wild type receptors ($P < 0.05$ and $P < 0.01$, respectively; one-way analysis of variance followed by the t-test modified according to Bonferroni). The residues mutated correspond to [van Rhee and Jacobson, 1995]: 131, 3.32; 132, 3.33; 226, 5.47; 277, 6.52; 280, 6.55; 307, 7.36; 310, 7.39.

Drug Dev Res. Author manuscript; available in PMC 2012 October 24.

TABLE 2

Blockade of 2-MeSADP-Induced Increases in [^{3}H]Inositol Phosphate Turnover by the P2-Receptor Antagonists MRS 2210, NF023, and Reactive Blue 2 at Wild Type and K280A Mutant P2Y$_1$-Receptors[a]

Antagonist	Wild type receptor			K280A mutant receptor		
	EC_{50} (2-MeSADP) [nM]	K_B (antagonist) [μM]	*n*	EC_{50} (2-MeSADP) [nM]	K_B (antagonist) [μM]	*n*
(control)	3.7±0.9	—	5	3,600±1,610	—	6
MRS 2210 (10 μM)	34.6±13.8 *	2.4±0.7	4	3,930±1,640	>10 [45±16 **][b]	4
NF023 (100 μM)	52.7±15.5 *	9.1±1.5	4	4,230±1,560 **	>100 [614±504] *[b]	4
Reactive blue 2 (10 μM)	48.2±20.5 *	1.6±0.6	4	7,600±4,920	11.3±2.7 **	3

[a] 2-MeSADP was applied at increasing concentrations for 30 min at 37°C. Antagonists or their solvent (control) was added 20 min before 2-MeSADP. EC_{50} values for 2-MeSADP were determined by fitting a sigmoid-shaped function to the data of each experiment. Experiments in the absence or presence of antagonists were performed in parallel. K_B values for antagonists were calculated from the respective increases in EC_{50} values. Means±S.E.M. of (*n*) experiments.

[b] The calculated estimates for the apparent K_B values [given in square brackets] are much higher than the applied concentrations of the antagonists.

* Statistically significant differences versus respective value in the absence of antagonists (control; $P < 0.05$; *t*-test).

* and *** Statistically significant differences vs. respective value in wild type receptors ($P < 0.05$ and $P < 0.01$, respectively; *t*-test).