

Research Article

Actions of a Series of PPADS Analogs at P2X₁ and P2X₃ Receptors

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Strategy, Management and Health Policy				
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ABSTRACT Seven PPADS (Pyridoxal-5'-Phosphate 6-Azophenyl 2',4'-DiSulfonate) analogs were investigated at Group I P2X receptors expressed in *Xenopus* oocytes. All seven analogs potentially inhibited P2X₁ (IC₅₀ range, 5–32 nM) and P2X₃ (IC₅₀ range, 22–345 nM), the two Group I P2X receptor subtypes. Analogs showed greater inhibitory activity where the pyridoxal moiety of PPADS contained a 5'-phosphonate group, rather than a 5'-phosphate group. Analogs also showed greater potency where disulfonate groups were removed from, or substituted at, the azophenyl moiety. The most active analog was MRS 2257 (pyridoxal-5'-phosphonate 6-azophenyl 3',5'-bismethylenephosphonate) at P2X₁ (IC₅₀, 5 nM) and P2X₃ (IC₅₀, 22 nM) receptors, being 14-fold and 10-fold more potent than PPADS itself. MRS 2257 produced a nonsurmountable inhibition when tested against a range of ATP concentrations, although blockade was reversed by about 85% after 20 minutes of washout. TNP-ATP and Ip₅l were equipotent with MRS 2257 at P2X₁ receptors, whereas TNP-ATP was 64-fold more potent than MRS 2257 at P2X₃ receptors. In conclusion, the PPADS template can be altered at the pyridoxal and phenyl moieties to produce P2X₁ and P2X₃ receptor antagonists showing higher potency and greater degree of reversibility than the parent compound at these Group I P2X receptors. *Drug Dev. Res.* 53:281–291, 2001. © 2001 Wiley-Liss, Inc.

Key words: purinoceptor; P2X receptor; ion channel; nucleotide; ATP; antagonist

INTRODUCTION

Adenosine 5'-triphosphate (ATP) is widely regarded as a major signalling molecule in the peripheral nervous system, where exocytotically released ATP can act on a plethora of P2 purinoceptor subtypes [Ralevic and Burnstock, 1998]. Purinergic receptors can be subdivided into two families: 1) P2X receptors gated principally by ATP, and incorporating an intrinsic ion-channel; and 2) P2Y receptors activated by either purine or pyrimidine nucleotides, and coupled to heterotrimeric G proteins. The P2X receptor family has been further subdivided into functional groups with distinct operational profiles [Khakh et al., 2001]. The first of these groups, Group I, includes the P2X₁ and P2X₃ receptors, which are characterised by exceedingly fast kinetics for ion channel activation and inactivation, an acute

sensitivity to the agonist α,β -methylene-ATP as well as ATP itself, and blockade by suramin and pyridoxal-5'-phosphate 6-azophenyl 2',4'-disulfonate (PPADS). P2X₁ receptors are

Abbreviations: ATP, adenosine-5'-triphosphate; Ip₅l, diinosine pentaphosphate; isoPPADS, pyridoxal-5'-phosphate 6-azophenyl 2',5'-disulfonate; MRS, Molecular Recognition Section; PPADS, pyridoxal-5'-phosphate 6-azophenyl 2',4'-disulfonate; P5P, pyridoxal-5'-phosphate; SAR, structure-activity relationship; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-ATP; V_h, holding potential.

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abundant in vascular smooth muscle, whereas P2X₃ receptors are concentrated in nociceptive sensory nerve fibres and their cell bodies [King, 1998; Ralevic and Burnstock, 1998; Khakh et al., 2001].

An awareness of the therapeutic importance of P2X₁ and P2X₃ receptors as drug targets has given impetus to the development of selective antagonists for these receptor subtypes [Burnstock, 1998; Williams and Jarvis, 2000]. PPADS was originally proposed to be a selective blocker of P2X receptors in vascular smooth muscle [Lambrecht et al., 1992; Ziganshin et al., 1993, 1994; McLaren et al., 1994]. Further studies revealed that, at high concentrations, PPADS blocked both native P2Y₁-like and recombinant P2Y₁ receptors [Boyer et al., 1994; Ziyal et al., 1994; Windscheif et al., 1994, 1995a,b; Brown et al., 1995; Schachter et al., 1996] and, at best, PPADS showed only a modest selectivity for P2X versus P2Y receptors. PPADS was also found to inhibit the hydrolytic activity of ecto-ATPase, thus enhancing ATP potency at both P2X and P2Y receptors and complicating the pharmacological analysis of PPADS antagonism in assay systems [Windscheif et al., 1995a; Chen et al., 1996; Ziganshin et al., 1996]. Such complications notwithstanding, PPADS does not noticeably interact with α_1 and β_1 adrenoceptors, muscarinic (M₁₋₄), histamine (H₁), serotonin (5-HT₃) or adenosine (A₁ and A_{2B}) receptors [Lambrecht et al., 1992, 2000; Boyer et al., 1994; Trezise et al., 1994a; Ziganshin et al., 1994], and, therefore, remains a useful template to design chemical derivatives with improved potency and selectivity at P2X receptor subtypes.

The first PPADS derivative investigated for altered potency was pyridoxal-5'-phosphate (P5P), which is a derivative of pyridoxine (vitamin B₆) and was reported to block P2X receptors in rat vagus nerve (pK_B, 5.4) and rat vas deferens (pK_B, 5.3) in a competitive manner [Trezise et al., 1994a]. Pyridoxine $\alpha^{4,5}$ -monophosphate (MRS 2219, a cyclized form of P5P) lost antagonist activity at P2X₁₋₄ receptors and potentiated ATP-responses at P2X₁ receptors [Jacobson et al., 1998]. IsoPPADS (pyridoxal-5'-phosphate 6-azophenyl 2',5'-disulfonate) was found to have similar potency to PPADS at P2X receptors in rat vagus nerve (pK_B, 6.0), although blockade by isoPPADS but not PPADS was reversible on washout [Trezise et al., 1994b]. IsoPPADS potently inhibited P2X₁ (IC₅₀, 43 nM) and P2X₃ (IC₅₀, 84 nM) receptors, whereas its cyclized form (pyridoxine $\alpha^{4,5}$ -monophosphate 6-azophenyl 2',5'-disulfonate; MRS 2220) showed a marked decrease in potency at P2X₁ (by 250-fold) and P2X₃ (by 700-fold) receptors [Jacobson et al., 1998]. The recently synthesised compound PPNDs (pyridoxal-5'-phosphate 6-(2'-naphthylazo-6'-nitro) 4',8'-disulfonate) showed a sixfold increase in potency over PPADS at P2X₁ receptors (pIC₅₀, 7.84) and P2X receptors in rat vas deferens (pK_B, 7.43) [Lambrecht et al., 2000].

During the last several years, the structure of PPADS derivatives has been gradually refined to improve their selectivity for P2X receptors [Jacobson et al., 1998, 1999; Kim et al., 1998, 2001]. Some 30 PPADS analogues have been synthesised and tested on a wide range of nucleotide receptors (P2X_{1,2,3,4,7} and P2Y_{1,2,4,6}), initially to identify compounds selective for P2X over P2Y receptors [Kim et al., 1998], and then to identify compounds with a higher potency at P2X₁ and P2X₃ receptors than other P2X subtypes [Kim et al., 2001]. Many of these improved PPADS analogues were still found to inhibit both ecto-ATPase and ecto-apyrase, but only when used at high micromolar concentrations [Hoffman et al., 2000; Ziganshin et al., 2000]. Thus, the present study investigated the antagonistic properties of a short series (7 of 30) of PPADS analogues that showed high selectivity for the Group I P2X receptor subtypes and low activity as inhibitors of ectoenzymes.

MATERIALS AND METHODS

Materials

All common salts were AnalaR grade (Aldrich Chemicals, Gillingham, UK). ATP (disodium salt) was purchased from Boehringer (Mannheim, Germany). Tricaine (3-amino-benzoic acid ethyl ester) and P5P were purchased from Sigma Chemical Co. (Poole, UK), PPADS from RBI (Natick, MA), isoPPADS from Tocris Cookson (St. Albans, UK), and 2',3'-O-trinitrophenyl-ATP (TNP-ATP) from Molecular Probes (Cambridge, UK). Diinosine pentaphosphate (Ip₅I) was a gift from Dr. Jesus Pintor (Madrid, Spain). The synthesis and purification of PPADS derivatives have been described elsewhere [Kim et al., 1998, 2001]. The preparation of carbon-bridged analogues has also been described previously [Kim and Jacobson, 2000]. Solutions of agonists and antagonists were prepared daily from stock solutions (10 or 100 mM, stored frozen) made up in extracellular bathing solution.

Oocyte Preparation and P2X Receptor Expression

Xenopus laevis were anaesthetised with Tricaine (0.2%, wt/vol) and killed by decapitation (in accordance with Institution regulations). The dissection and removal of ovaries, as well as the preparation of defolliculated *Xenopus* oocytes, have been described in detail elsewhere [King et al., 1997]. Defolliculated oocytes do not possess native P1 or P2 receptors that could otherwise complicate the analysis of agonist activity [King et al., 1996a,b]. Also, defolliculated oocytes are largely devoid of ecto-ATPase activity, so avoiding the complicating issue of ectoenzyme inhibition by P2 receptor antagonists [Ziganshin et al., 1995]. Mature oocytes (stages V and VI) were injected (40 nl) cytosolically with capped ribonucleic acid (cRNA, 1 mg/ml) encoding either rat P2X₁ or rat P2X₃ receptor subunits. Injected oocytes were incubated

at 18°C in a bathing solution (pH 7.5) containing (mM): NaCl 110, KCl 1, NaHCO₃ 2.4, Tris-HCl 7.5, Ca(NO₃)₂ 0.33, CaCl₂ 0.41, MgSO₄ 0.82, supplemented with gentamycin sulphate 50 µg/l for 48 h to allow full receptor expression, then stored at 4°C for up to 12 days.

Electrophysiology

Nucleotide-evoked membrane currents were recorded from cRNA-injected oocytes studied under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2B; Foster City, CA). Intracellular microelectrodes had a resistance of 1–2 MΩ when filled with KCl (3 M). Oocytes were perfused constantly (at 5 ml min⁻¹) with an extracellular solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, BaCl₂ 1.8, pH 7.4–7.5. All recordings were made at room temperature (18°C) at a holding potential between –60 and –90 mV. Electrophysiological data were filtered initially at 3 kHz, captured at a rate of 20 Hz on a computer connected to an MP100WSW interface (Biopac Systems, Inc., Goleta, CA) and displayed using commercial software (Acqknowledge III, Biopac).

Compound Solutions

Solutions were delivered by gravity flow from independent reservoirs placed above the recording chamber (volume, 0.5 ml). ATP was applied for 30 sec and, thereafter, washed off for a period of 20 min with extracellular bathing solution. P2 receptor antagonists were applied 20 min before, and during, agonist application. Only one antagonist was tested in each experiment. IC₅₀ values were determined from Hill plots, using the transform $\log(I/I_{\max}-I)$ where I was the current evoked by the agonist (used at the EC₇₀ concentration). For agonist concentration-response (C/R) curves, data were normalised to the maximum agonist response (I_{\max}) to ATP (300 µM) at pH 7.5. Two C/R curves were produced in each experiment, before and after the addition of one concentration of antagonist. EC₅₀ and EC₇₀ values were determined from Hill plots, using the transform $\log(I/I_{\max}-I)$ where I was the current evoked by each concentration of agonist. The Hill coefficient was taken from the slope of Hill plots. C/R curves and inhibition curves were fitted by nonlinear regression analysis using commercial software (Prism v2.0, GraphPad; San Diego, CA).

Statistics

Data are presented as mean ± s.e.m. of four sets of data from different batches of oocytes. Student's unpaired t test was used and P values ≤ 0.05 were considered significant.

RESULTS

The Group I P2X receptor-selective antagonists used in this study were PPADS analogues possessing pyridoxal-

5'-phosphate or pyridoxal-5'-phosphonate moieties, the structures of which are shown in Figure 1. The code name, formula, molecular weight, and chemical name of seven key analogues are listed in Table 1. The blocking activity of PPADS, isoPPADS, the related analogues, and two known standards (Ip₅I and TNP-ATP) were tested over a wide range of concentrations (0.01–30,000 nM) against ATP-evoked inward currents at either P2X₁ or P2X₃ receptors. The resultant inhibition curves for a total of 11 P2 receptor antagonists acting at each P2X subtype are shown in Figure 2. Mean IC₅₀ values, 95% confidence intervals, and Hill coefficients are given in Table 2. The most potent PPADS analogue was MRS 2257 at P2X₁ receptors (mean IC₅₀, 5 nM) and P2X₃ receptors (mean IC₅₀, 22 nM). The route to its discovery is described below.

Structure Activity Relationship of PPADS Analogues

In the present study, PPADS was an effective antagonist at submicromolar concentrations and approximately threefold more potent at P2X₁ than P2X₃ receptors (mean IC₅₀, 68 versus 214 nM). The isoform, isoPPADS, was also a potent antagonist at P2X₁ and P2X₃ receptors (mean IC₅₀, 35 versus 79 nM). Thus, isoPPADS was 2- or 3-fold more potent than PPADS at the Group I P2X receptors. MRS 2191, a 5'-phosphonate derivative of isoPPADS, was even more potent than isoPPADS at P2X₁ receptors (mean IC₅₀, 9 versus 35 nM), although markedly less potent than isoPPADS at P2X₃ receptors (mean IC₅₀, 229 versus 79 nM).

MRS 2143, a 5'-phosphate derivative lacking sulfonate groups on the azophenyl moiety, was twofold more potent than PPADS at P2X₁ receptors (mean IC₅₀, 32 versus 68 nM) and slightly less potent than PPADS at P2X₃ receptors (mean IC₅₀, 345 versus 214 nM). However, this compound revealed to us that effective antagonism did not require highly polar sulfonate groups on the azophenyl moiety per se, an advantage in drug design.

A carboxylate group was added at various positions on the azophenyl moiety of the MRS 2143 template, and the most interesting compound was MRS 2159. This compound increased antagonist potency at both P2X receptor subtypes and, consequently, MRS 2159 was sevenfold more potent than PPADS at P2X₁ receptors (mean IC₅₀, 9 versus 68 nM) and 2-fold more potent than PPADS at P2X₃ receptors (mean IC₅₀, 118 versus 214 nM). Conversion of MRS 2159 into a 5'-phosphonate compound (MRS 2284) retained antagonist potency at P2X₁ and P2X₃ receptors (mean IC₅₀, 7 and 139 nM). However, the addition of a second carboxylate group to MRS 2284, to produce MRS 2285, had a deleterious effect and reduced antagonist potency at P2X₁ and, more so, P2X₃ receptors (mean IC₅₀ values, 22 and 260 nM).

Concentrating on pyridoxal-5'-phosphonate analogues, the effects of different substitutions at the azophenyl moiety were explored by replacing sulfonate and carboxy-

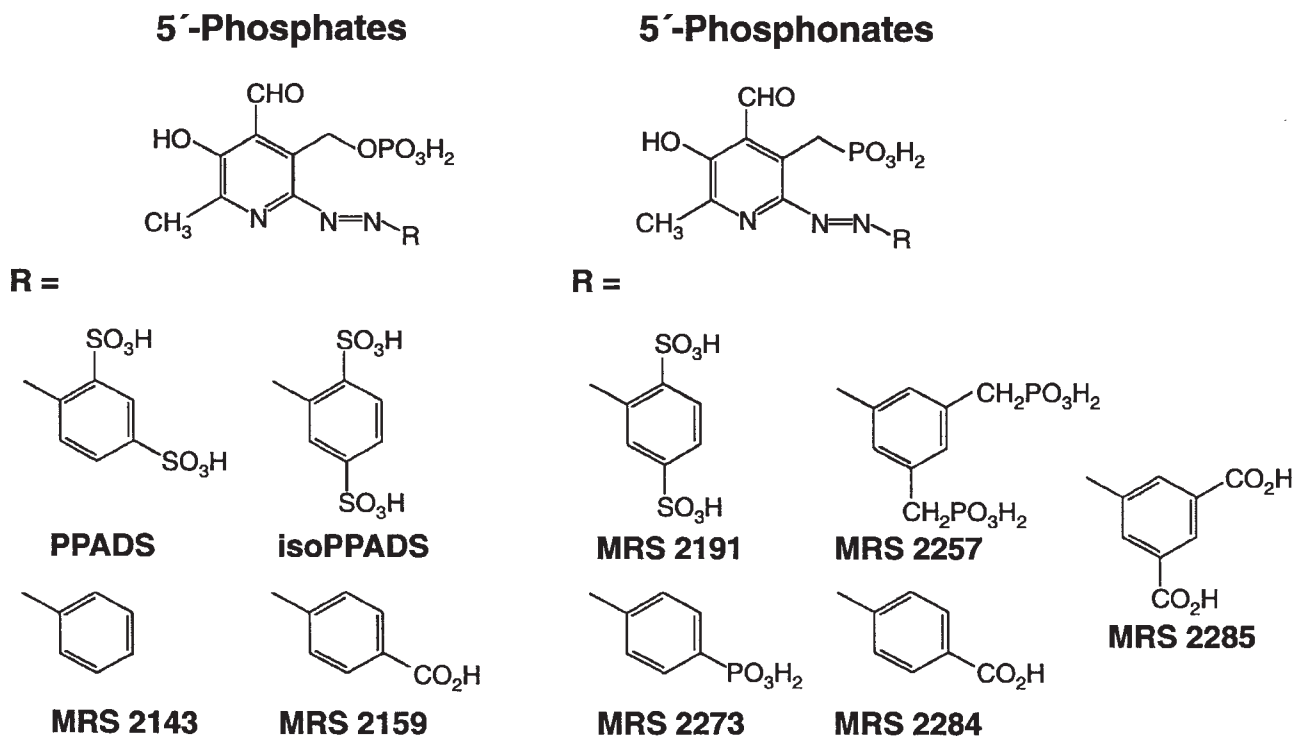


Fig. 1. Chemical structure of PPADS and related analogues. The analogues used in this study were based on pyridoxal-5'-phosphates (left) and pyridoxal-5'-phosphonates (right). Structurally related derivatives of

each group were synthesised and tested for pharmacological activity at P2X₁ and P2X₃ receptors.

late groups with either phosphonate or methylphosphonate groups. MRS 2273 (phosphonate added) and MRS 2257 (methylene-phosphonates added) showed a marked increase in potency, particularly at P2X₁ receptors at which mean IC₅₀ values of 11 nM and 5 nM were seen for these two compounds. MRS 2273 was sixfold, and MRS 2257 14-fold, more potent than PPADS at P2X₁ receptors. These derivatives were also effective antagonists at P2X₃ receptors (mean IC₅₀, 33 and 22 nM), being sixfold and 10-fold more potent than PPADS at P2X₃ receptors.

The Azo (-N=N-) Linkage

PPADS and isoPPADS slowly decompose in aqueous solution when exposed to white light at room temperature because the azo (-N=N-) bridge connecting the

pyridoxal and phenyl moieties is chemically unstable (G. Semple, personal communication). Therefore, two pilot studies of new PPADS analogues were initiated to test the effects of changing the azo-linkage in the sulfonate-free analogue MRS 2143 (Fig. 3A,B and Table 3). In the first pilot study, MRS 2143 was converted first into a pyridoxal bisphosphate analogue (MRS 2260) and, thereafter, the azo-linkage replaced by an ethene (-C=C-) bridge in MRS 2259. The antagonist activity of MRS 2260 at P2X₁ receptors was significantly reduced (~53% blockade at 10 μM), whereas the activity of MRS 2259 was not appreciably lower (~40% blockade at 10 μM). In a second pilot study, substitution of the azo bridge in MRS 2143 with a methylene (CH₂) bridge maintained the inhibitory activity of MRS 2335 (mean IC₅₀, 37 nM) which

TABLE 1. PPADS and Related Analogues

Compound	Formula	Mol. wt.	Name
PPADS	C ₁₄ H ₁₀ N ₃ O ₁₂ PS ₂ Na ₄	599.3	Pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonate
isoPPADS	C ₁₄ H ₁₀ N ₃ O ₁₂ PS ₂ Na ₄	599.3	Pyridoxal-5'-phosphate-6-azophenyl-2',5'-disulfonate
MRS 2143	C ₁₄ H ₁₂ N ₃ O ₆ PNa ₂	395.2	Pyridoxal-5'-phosphate-6-azophenyl
MRS 2159	C ₁₅ H ₁₃ N ₃ O ₈ PNa	417.2	Pyridoxal-5'-phosphate-6-azophenyl-4'-carboxylate
MRS 2191	C ₁₄ H ₁₄ N ₃ O ₁₁ PS ₂ Na ₃	561.3	Pyridoxal-5'-phosphonate-6-azophenyl-2',5'-disulfonate
MRS 2257	C ₁₆ H ₁₈ N ₃ O ₁₁ P ₃ Na ₃	590.2	Pyridoxal-5'-phosphonate-6-azophenyl-3',5'-bismethylenephosphonate
MRS 2273	C ₁₄ H ₁₃ N ₃ O ₈ P ₂ Na ₂	459.2	Pyridoxal-5'-phosphonate-6-azophenyl-4'-phosphonate
MRS 2284	C ₁₅ H ₁₄ N ₃ O ₇ PNa ₁	402.2	Pyridoxal-5'-phosphonate-6-azophenyl-4'-carboxylate
MRS 2285	C ₁₆ H ₁₄ N ₃ O ₉ PNa ₃	443.3	Pyridoxal-5'-phosphonate-6-azophenyl-3',5'-dicarboxylate

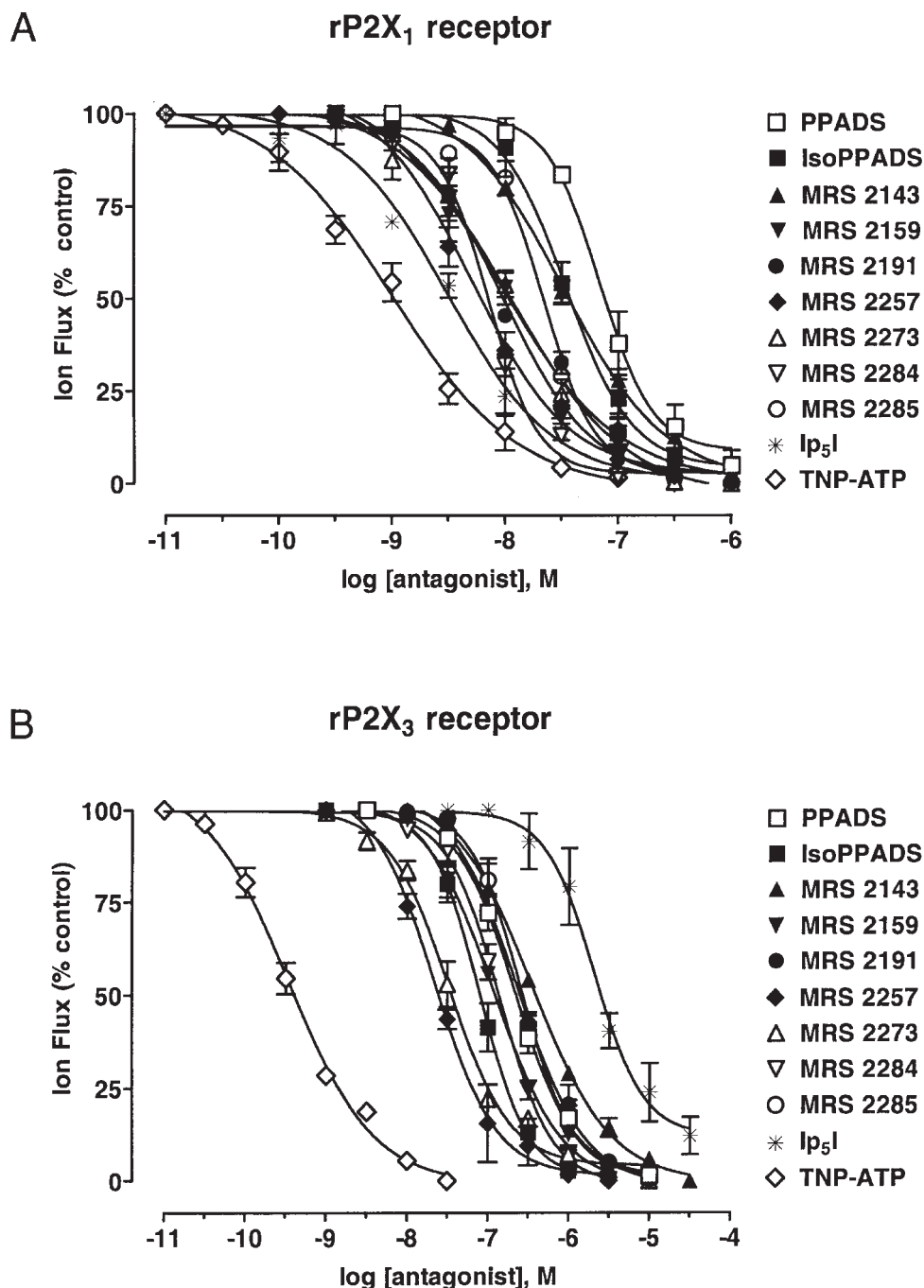


Fig. 2. Blocking activity of PPADS and related compounds. Inhibition curves for PPADS, isoPPADS, seven related PPADS-like compounds, and two standards (Ip₅I and TNP-ATP) at P2X₁ receptors (**A**) and P2X₃ recep-

tors (**B**). P2X receptors were activated by ATP at approximately the EC₇₀ concentration (P2X₁, 1 μM; P2X₃, 3 μM). Data represent mean ± SEM for four determinations from separate batches of *Xenopus* oocytes.

was equipotent with MRS 2143 and twofold more potent than PPADS. A comparison of the pyridoxal-5'-phosphate compound (MRS 2335) with the pyridoxal-5'-phosphonate derivative (MRS 2305) revealed a reduction in blocking activity (mean IC₅₀, 214 nM), MRS 2305 being markedly less potent than either MRS 2143 (by sixfold) or PPADS (by threefold).

Antagonism by TNP-ATP and Ip₅I

Two recently identified antagonists, with high potency at P2X₁ and P2X₃ receptors, were also tested as standards to provide a comparative index of antagonism. Nanomolar concentrations of trinitrophenyl-ATP (TNP-ATP) blocked both P2X₁ and P2X₃ receptors (mean IC₅₀, 1 and 0.34 nM), this compound showing threefold higher

TABLE 2. Antagonist Activity at Group I P2X Receptors

Compound	rP2X ₁			rP2X ₃		
	IC ₅₀ (nM)	CI (nM)	n _H	IC ₅₀ (nM)	CI (nM)	n _H
PPADS	68.5	45.9 – 102.2	–1.87	213.6	173.0 – 263.7	–1.17
isoPPADS	34.5	25.3 – 47.1	–1.48	78.8	57.4 – 108.0	–1.46
MRS 2143	32.4	26.5 – 40.4	–0.97	345.3	240.6 – 495.4	–0.88
MRS 2159	9.4	6.6 – 13.4	1.08	117.7	93.4 – 148.3	–1.02
MRS 2191	8.9	5.9 – 13.6	–0.84	228.6	171.0 – 305.5	–1.01
MRS 2257	5.1	3.6 – 7.3	–1.02	21.8	13.8 – 34.5	–1.13
MRS 2273	11.3	7.5 – 17.1	–0.86	32.9	25.8 – 41.9	–1.15
MRS 2284	7.1	6.0 – 8.4	–1.98	139.0	114.1 – 169.3	–1.33
MRS 2285	21.9	17.4 – 27.5	–1.9	259.8	222.3 – 303.8	–1.34
Ip ₅ I	3.13	2.09 – 4.69	–0.94	2059.0	1252.0 – 3388.0	–1.43
TNP-ATP	1.01	0.69 – 1.47	–0.78	0.34	0.25 – 0.46	–0.82

Each compound was tested against ATP-responses (rP2X₁, 1 μ M; rP2X₃, 3 μ M). Antagonist activity is expressed as mean IC₅₀ value (nM, for four determinations) with confidence intervals for the mean at the 95% level (CI, nM). The mean Hill coefficient (n_H) is also given for each set of inhibition curves.

potency at P2X₃ receptors. Diinosine pentaphosphate (Ip₅I) was found to be highly selective for P2X₁ receptors (mean IC₅₀, 3 nM), and 700-fold less potent at P2X₃ receptors (mean IC₅₀, 2059 nM). Against the most potent PPADS analogue, these two standards were approximately equipotent with MRS 2257 at P2X₁ receptors, whereas TNP-ATP was 64-fold more potent than MRS 2257 at P2X₃ receptors.

Reversibility of P2X Receptor Antagonism

The ability of P2X₁ and P2X₃ receptors to recover from blockade was assessed 20 min after washout of a maximally effective blocking concentration of each PPADS analogue. Full recovery of agonist responses was observed after isoPPADS washout (P2X₁, 101 \pm 4%; P2X₃, 105 \pm 5%; mean \pm SEM, n = 3), and partial recovery observed after PPADS washout (P2X₁, 31 \pm 10%; P2X₃, 44 \pm 7%). MRS 2143, the PPADS analogue lacking sulfonate groups, also showed a reasonable level of recovery (P2X₁, 74 \pm 5%; P2X₃, 79 \pm 6%). Additionally, MRS 2257 showed a good level of recovery (P2X₁, 82 \pm 5%; P2X₃, 85 \pm 4%). The carboxylated 5'-phosphonate compounds (MRS 2284 and MRS 2285) each caused a near irreversible blockade (<25% recovery).

Nature of Antagonism

The nature of antagonism caused by PPADS-like derivatives was assessed from C/R curves for ATP, in the absence and presence of each compound. PPADS and isoPPADS antagonism were first assessed at P2X₁ receptors (Fig. 4) and P2X₃ receptors (Fig. 5). Thereafter, two additional compounds were investigated—MRS 2257 (a potent and slowly reversible antagonist) and MRS 2284 (a potent and irreversible antagonist). Each analogue exerted a nonsurmountable antagonism at P2X₁ and P2X₃ receptors (Figs. 4 and 5). There was no significant change

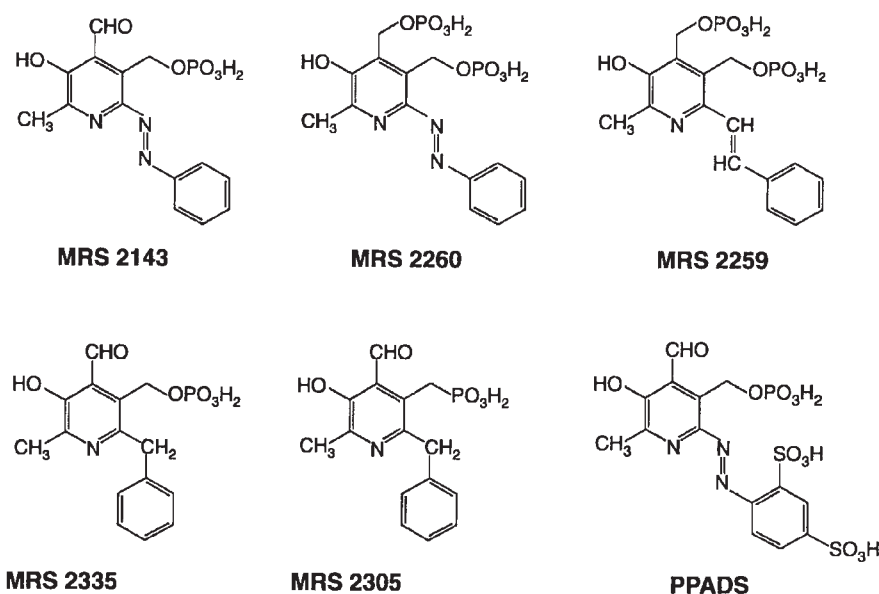
in the EC₅₀ values for ATP in the presence of each compound (data not shown), although the maximum agonist response was progressively decreased in a concentration-dependent manner.

DISCUSSION

In the present study, the antagonist activity of a short series of PPADS analogues at P2X₁ and P2X₃ receptors—the Group I P2X receptor subtypes—was examined. Seven analogs were selected from a library of 30 PPADS derivatives on the grounds that, except for P2X₁ and P2X₃ receptors, these compounds did not show significantly different blocking activity to PPADS at other P2X and P2Y receptor subtypes [Kim et al., 2001]. Also, these seven compounds showed low inhibitory activity at surface enzymes capable of breaking down ATP [Hoffman et al., 2000; Ziganshin et al., 2000]. All seven analogs were shown here to be effective inhibitors of ATP-mediated inward currents at P2X₁ and P2X₃ receptors. At P2X₁, IC₅₀ values ranged from 5 to 22 nM, and the best compound, MRS 2257, was 14-fold more potent than PPADS. At P2X₃, IC₅₀ values ranged from 22 to 345 nM and the best compound, again MRS 2257, was 10-fold more potent than PPADS. The high potency of the methylene-phosphonate analogue, MRS 2257, at P2X₁ and P2X₃ receptors may be related to the avidity of Group I P2X receptors for the methylene-phosphonate based agonists, α , β -meATP and β , γ -meATP.

One drawback to the use of PPADS as an antagonist at P2 receptors is the apparent irreversibility of blockade. In the present study, some of the tested analogs were found to reverse slowly and, as pointed out by Lambrecht et al. [1992], the term *pseudo-irreversible blockade* is probably more appropriate. Blockade by the most potent analogue, MRS 2257, was relaxed by approximately 82–85% after 20-min washout and this com-

A.



B.

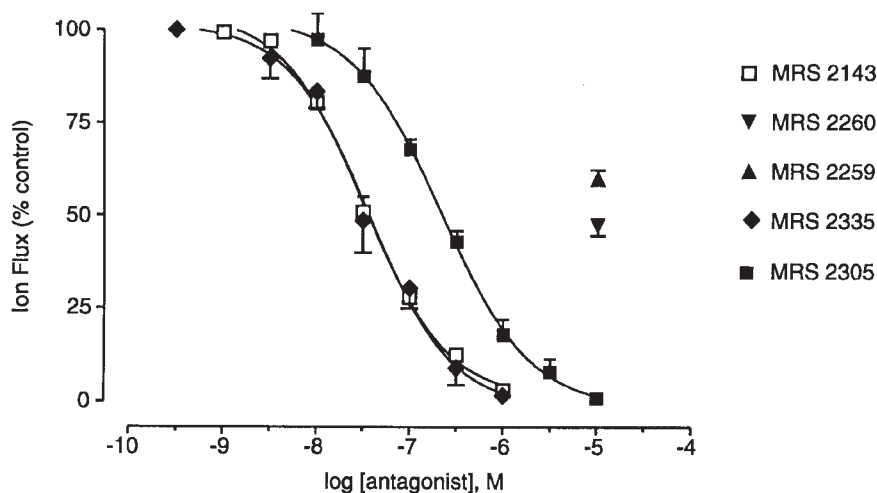


Fig. 3. Effect of azo-linkage on antagonist activity. In **(A)**, chemical structure of compounds related to a nonsulfonated derivative of PPADS (MRS 2143) showing variations in the azo-phenyl linkage. In **(B)**, inhibition curves for MRS 2143, MRS 2335, and MRS 2305 at P2X₁ receptors activated by

ATP (1 μ M). MRS 2259 and MRS 2260 were tested at a single concentration (10 μ M). Data represent mean \pm SEM for four determinations from separate batches of *Xenopus* oocytes.

pared favourably against 31–44% relaxation after PPADS. Blockade by isoPPADS was fully relaxed after 20-min washout. However, analysis of the C/R curves for ATP showed that blockade by MRS 2257, PPADS, and isoPPADS was nonsurmountable in each case. This observation suggests that the off-rate of these antagonists is significantly slower than either the on-rate for the ago-

nist or the desensitisation rate at these rapidly inactivating P2X receptors.

We also compared the blocking activity of MRS 2257 against two known standards, Ip₅I and TNP-ATP, which currently represent the most potent antagonists at Group I P2X receptors [Virginio et al., 1998; King et al., 1999]. By statistical analysis, MRS 2257 was as potent as

TABLE 3. Antagonist Activity at P2X₁ Receptor

Compound	IC ₅₀ (nM) or % inhibition	CI (nM)	n _H
PPADS	68.5	45.9 – 102.2	-1.87
MRS 2143	32.4	26.5 – 40.4	-0.97
MRS 2335	36.9	22.7 – 60.1	-0.98
MRS 2305	213.7	127.9 – 357.2	-0.90
MRS 2259 (10 μM)	40 ± 3%	—	nd ^a
MRS 2260 (10 μM)	53 ± 3%	—	nd ^a

Each compound was tested against P2X₁ activated by ATP (1 μM). Antagonist activity is expressed as mean IC₅₀ value (nM, for four determinations) with confidence intervals for the mean at the 95% level (CI, nM) or, where tested at a single concentration (10 μM), as a percentage inhibition of ATP responses (for four determinations). The mean Hill coefficient (n_H) is also given for each set of inhibition curves. ^and, not determined.

Ip₅I and TNP-ATP at P2X₁ receptors, whereas MRS 2257 was 64-fold less potent than TNP-ATP at P2X₃ receptors. The activity of Ip₅I and TNP-ATP is about 300- to 1,000-fold lower at native P2X receptors *in vivo*, because each of these nucleotidic compounds is broken down by tissue enzymes [Hoyle et al., 1997; Lewis et al., 1998]. The antagonist actions of MRS 2257 have not yet been examined in whole tissues because of production problems in generating sufficient quantities of this compound.

The observed blocking activity of the seven tested analogues, PPADS, and isoPPADS has helped in understanding the structural requirements for potent antagonists at Group I P2X receptors. In this study, azo-linked analogues with pyridoxal-5'-phosphonate moieties were found to be more potent than their pyridoxal 5'-phos-

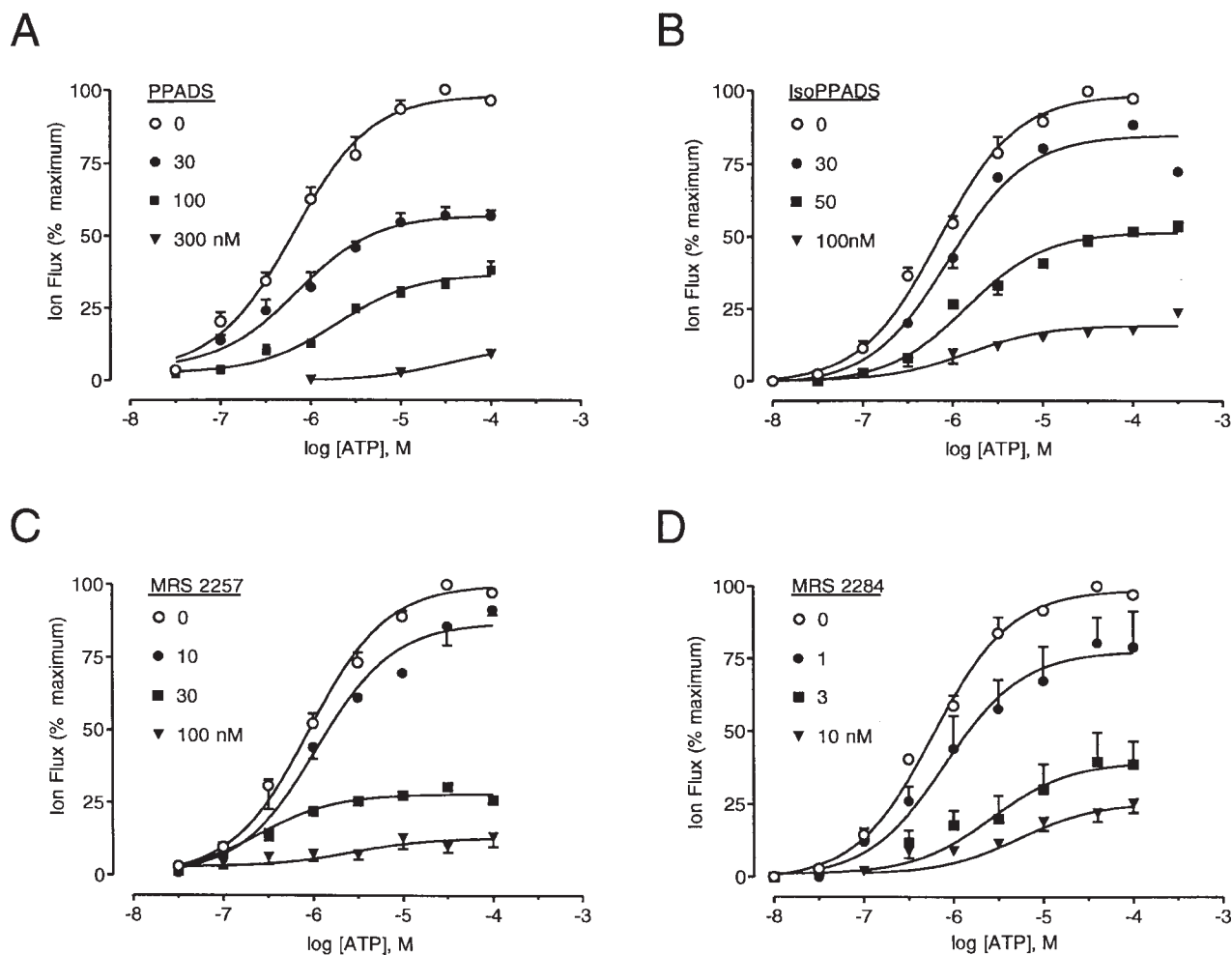


Fig. 4. A–D: Nature of antagonist blockade at P2X₁ receptors. Concentration/response (C/R) relationship for ATP, in absence and presence of the P2X₁ receptor antagonists. Paired C/R curves (control and test) were obtained for each concentration of antagonist studied. Data (mean ± SEM,

n = 4) are expressed as a percentage of the maximum ATP-response (at pH 7.5) before the addition of antagonist. Where missing, error bars are smaller than symbol size.

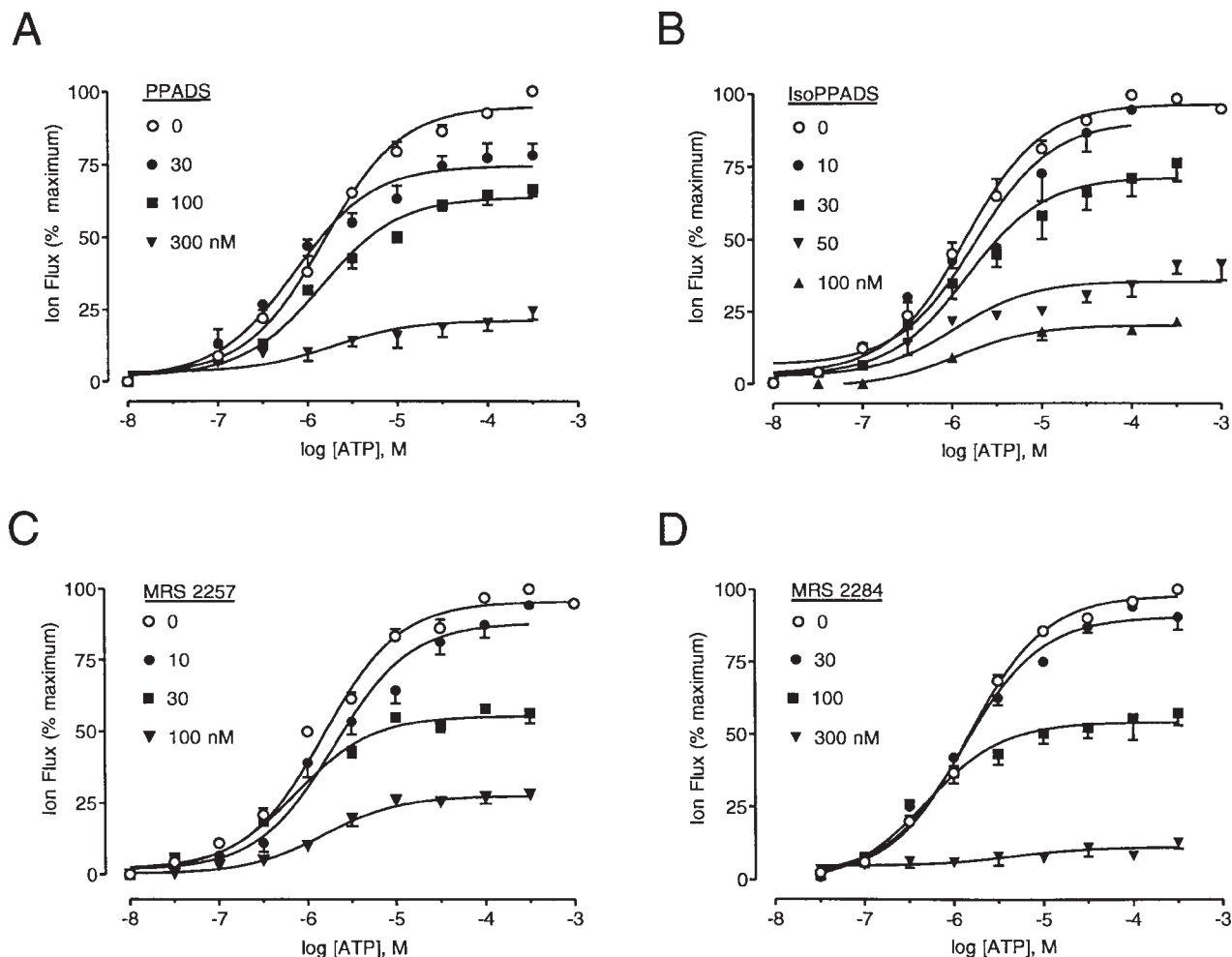


Fig. 5. Nature of antagonist blockade at P2X₃ receptors. Concentration/response (C/R) relationship for ATP, in absence and presence of the P2X₃ receptor antagonists. Paired C/R curves (control and test) were obtained for each concentration of antagonist studied. Data (mean \pm SEM, $n = 4$)

are expressed as a percentage of the maximum ATP-response (at pH 7.5) before the addition of antagonist. Where missing, error bars are smaller than symbol size.

phate counterparts. It was also clear that the pyridoxal bis-4',5'-phosphate derivatives MRS 2259/2260 (shown in Fig. 3) were markedly less potent than PPADS and related analogues at Group I P2X receptors. These present results complement our earlier findings where the cyclic pyridoxine- $\alpha^{4,5}$ -phosphate derivatives of P5P (MRS 2219) and isoPPADS (MRS 2200) showed diminished or no antagonist activity at Group I P2X receptors [Jacobson et al., 1998]. Others have shown that 5'-dephosphorylated pyridoxine hydrochloride is less potent than pyridoxal 5'-phosphate at P2X receptors in rat vas deferens [Trezise et al., 1994a].

When modifications to the azophenyl moiety were analysed, it was clear that PPADS analogues could either dispense with disulfonate groups (e.g., MRS 2143) or accommodate other substitutions and still retain antagonist activity at P2X₁ receptors. Our present findings showed that azophenyl moieties with carboxylate (MRS

2159, 2284, 2285), phosphate (MRS 2273), and methylene-phosphonate (MRS 2257) substitutions yielded highly potent antagonists for the P2X₁ receptor. Closer inspection of inhibition data showed that desulfonated MRS 2143 and carboxylated MRS 2285 were less potent than PPADS at P2X₃ receptors, in contrast to their actions at P2X₁ receptors. This observation suggests that separate lines of chemistry are possible for P2X₁ and P2X₃ receptor-selective antagonists, although we have some way to go to improve the selectivity window or specificity for one P2X subtype over the other. Apart from the above modifications, others have shown that the azophenyl moiety can be replaced by naphthylazo groups (e.g., PPNDs) and improve antagonist potency at P2X₁ receptors [Lambrecht et al., 2000].

PPADS will break down in solution, at room temperature and under white light, because of the chemical reactivity of the azo (-N=N-) linkage (G. Semple, per-

sonal communication). Accordingly, we have begun to explore ways of dealing with this instability by replacing the azo bridge with other linkages. The conversion of the azo bridge, in MRS 2143, to a methylene bridge, in MRS 2235, caused no significant change in the blocking activity at P2X₁ receptors. This observation has prompted us to produce a new series of derivatives based on MRS 2235, with various additions to the benzyl moiety, to improve activity and selectivity at the Group I P2X receptors. Our results with ethene-bridged compounds related to MRS 2259 (Fig. 3) were also encouraging, and we are exploring synthesis routes for like analogues. We have yet to test either of these new series on a full range of P2X and P2Y receptor subtypes.

In summary, the present results have shown that the PPADS template remains amenable to chemical modification and can yield highly potent antagonists for Group I P2X receptors. Some analogues were more potent at P2X₁ receptors and, to this extent, we are starting to see the beginnings of selectivity for this receptor subtype. The scope for modification is not yet exhausted, and we hope to improve on the current indices of drug activity by altering the molecule in three positions (pyridoxal and azophenyl moieties and azo linkage). However, the current series of antagonists represent a significant improvement on PPADS—the best compound, MRS 2257, being at least 10-fold more potent at Group I P2X receptors and its blocking actions reversible with washout.

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