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## Structure–Activity Relationships of Pyridoxal Phosphate Derivatives as Potent and Selective Antagonists of P2X<sub>1</sub> Receptors

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Novel analogues of the P2 receptor antagonist pyridoxal-5'-phosphate 6-azophenyl-2',5'-disulfonate (**2**) were synthesized and studied as antagonists in functional assays at recombinant rat P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptors expressed in *Xenopus* oocytes (ion flux stimulation) and at turkey erythrocyte P2Y<sub>1</sub> receptors (phospholipase C activation). Selected compounds were also evaluated as antagonists of ion flux and the opening of a large pore at the recombinant human P2X<sub>7</sub> receptor. Modifications were made in the 4-aldehyde and 5'-phosphate groups of the pyridoxal moiety: i.e. a CH<sub>2</sub>OH group at the 4-position in pyridoxine was either condensed as a cyclic phosphate or phosphorylated separately to form a bisphosphate, which reduced potency at P2 receptors. 5-Methylphosphonate substitution, anticipated to increase stability to hydrolysis, preserved P2 receptor potency. At the 6-position, halo, carboxylate, sulfonate, and phosphonate variations made on the phenylazo ring modulated potency at P2 receptors. The *p*-carboxyphenylazo analogue, **4**, of phosphate **2** displayed an IC<sub>50</sub> value of 9 nM at recombinant P2X<sub>1</sub> receptors and was 1300-, 16-, and > 10000-fold selective for P2X<sub>1</sub> versus P2X<sub>2</sub>, P2X<sub>3</sub>, and P2Y<sub>1</sub> subtypes, respectively. The corresponding 5-methylphosphonate was equipotent at P2X<sub>1</sub> receptors. The 5-methylphosphonate analogue containing a 6-[3,5-bis(methylphosphonate)]-phenylazo moiety, **9**, had IC<sub>50</sub> values of 11 and 25 nM at recombinant P2X<sub>1</sub> and P2X<sub>3</sub> receptors, respectively. The analogue containing a phenylazo 4-phosphonate group, **11**, was also very potent at both P2X<sub>1</sub> and P2X<sub>3</sub> receptors. However, the corresponding 2,5-disulfonate analogue, **10**, was 28-fold selective for P2X<sub>1</sub> versus P2X<sub>3</sub> receptors. None of the analogues were more potent at P2X<sub>7</sub> and P2Y<sub>1</sub> receptors than **2**, which acted in the micromolar range at these two subtypes.

### Introduction

Adenine and uracil 5'-nucleotides act in cellular signaling in the nervous, muscular, cardiovascular, renal, and immune systems, through the activation of P2 receptors.<sup>1–3</sup> These nucleotide receptors consist of two families of distinct structure and function: P2X ligand-gated cation channels and G protein-coupled P2Y receptors.<sup>4</sup> P2X<sub>1–7</sub> and P2Y<sub>1,2,4,6,11</sub> designations have been unambiguously assigned to mammalian nucleotide receptors,<sup>5–7</sup> although there is still uncertainty about the correspondence of these sequences to the pharmacological phenotypes described prior to P2 receptor cloning.<sup>8</sup>

The therapeutic potential of potent and selective agonists and antagonists of P2 receptors has been discussed, although in mainly hypothetical terms due to the current lack of such agents.<sup>9</sup> For example, activation of the P2X<sub>1</sub> subtype mediates vasoconstriction at vascular smooth muscle, thus selective antagonists

may be antihypertensive. The P2Y<sub>1</sub> subtype participates in blood platelet aggregation, thus selective antagonists may be useful in regulating hemostasis. It appears that activation of the P2X<sub>3</sub> receptor subtype mediates nociception via the dorsal root ganglia, thus a selective antagonist may be antinociceptive.<sup>10,11</sup> Activation of the P2X<sub>7</sub> receptor subtype induces apoptosis in the immune and inflammatory system, thus selective antagonists may also be useful therapeutically.<sup>12</sup>

Derivatives of pyridoxal-5'-phosphate in which an azoaryl group is present at the 6-position were shown to be nonselective P2 receptor antagonists.<sup>13</sup> Compounds **1** (pyridoxal- $\alpha$ -5'-phosphate-6-azophenyl-2',4'-disulfonic acid) and **2** (the 2,5-disulfonate isomer) (Figure 1) have been studied as antagonists of adenine nucleotides at the turkey erythrocyte P2Y<sub>1</sub> receptor<sup>14,15</sup> and at P2X<sub>1</sub>-like receptors in the rabbit vas deferens,<sup>16</sup> urinary bladder,<sup>17</sup> isolated blood vessels,<sup>18</sup> guinea-pig isolated vas deferens,<sup>19</sup> and perfused rat mesenteric arterial bed.<sup>20</sup> **1** was also found to be a weak antagonist at the P2X<sub>7</sub> receptor in mouse microglial cells.<sup>21</sup> Both **1** and **2** have been shown to be antagonists of ATP (adenosine-5'-triphosphate) at P2X<sub>3</sub>-like receptors in sensory neurons.<sup>22–24</sup>

The present study demonstrates that both subtype

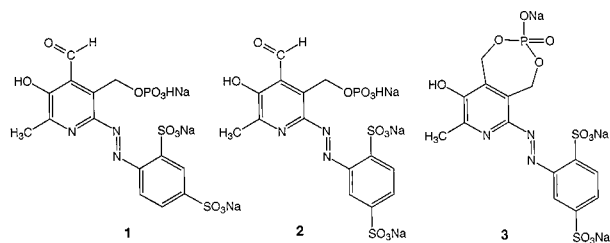
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**Figure 1.** Structures of azo derivatives of pyridoxal-5'-phosphate (**1** and **2**) and a cyclic pyridoxine- $\alpha^{4,5}$ -monophosphate derivative (**3**), all of which act as P2 receptor antagonists.

**Table 1.** MS and HPLC<sup>a</sup> Characterization of the Pyridoxal-Phosphate Analogues Synthesized

no.	formula	% yield	FAB (M - H <sup>+</sup> )		HPLC (t <sub>R</sub> , min)	
			calcd	found	A	B
5	C <sub>15</sub> H <sub>14</sub> N <sub>3</sub> O <sub>7</sub> P	71	378.0491	378.0494	4.6	10.50
7	C <sub>16</sub> H <sub>14</sub> N <sub>3</sub> O <sub>9</sub> P	59	422.0389	422.0383	5.2	12.9
9	C <sub>16</sub> H <sub>20</sub> N <sub>3</sub> O <sub>11</sub> P <sub>3</sub>	21	522.0232	522.0221	5.2	12.4
11	C <sub>14</sub> H <sub>14</sub> N <sub>3</sub> NaO <sub>8</sub> P <sub>2</sub>	68	436.0076	436.0071	5.5	10.0
13	C <sub>14</sub> H <sub>12</sub> ClN <sub>3</sub> NaO <sub>8</sub> PS	59	469.9591	469.9608	10.5	12.8
14	C <sub>15</sub> H <sub>14</sub> N <sub>3</sub> O <sub>7</sub> P	43	378.0491	378.0481	8.2	10.2
15	C <sub>16</sub> H <sub>14</sub> N <sub>3</sub> NaO <sub>12</sub> PS <sub>2</sub>	100	579.9474	579.9475	7.2	13.4
16	C <sub>14</sub> H <sub>14</sub> N <sub>3</sub> O <sub>5</sub> P	28	334.0593	334.0607	7.2	10.1
17	C <sub>8</sub> H <sub>13</sub> NO <sub>3</sub> P <sub>2</sub>	23	327.9987	328.0004	4.5	8.7
18	C <sub>14</sub> H <sub>15</sub> N <sub>3</sub> NaO <sub>15</sub> P <sub>2</sub> S <sub>2</sub>	80	635.9137	635.9103	5.5	17.1
19	C <sub>14</sub> H <sub>16</sub> ClN <sub>3</sub> O <sub>12</sub> P <sub>2</sub> S	73	545.9540	545.9545	7.8	14.3
20	C <sub>14</sub> H <sub>17</sub> N <sub>3</sub> O <sub>9</sub> P <sub>2</sub>	81	432.0362	432.0339	6.7	12.8

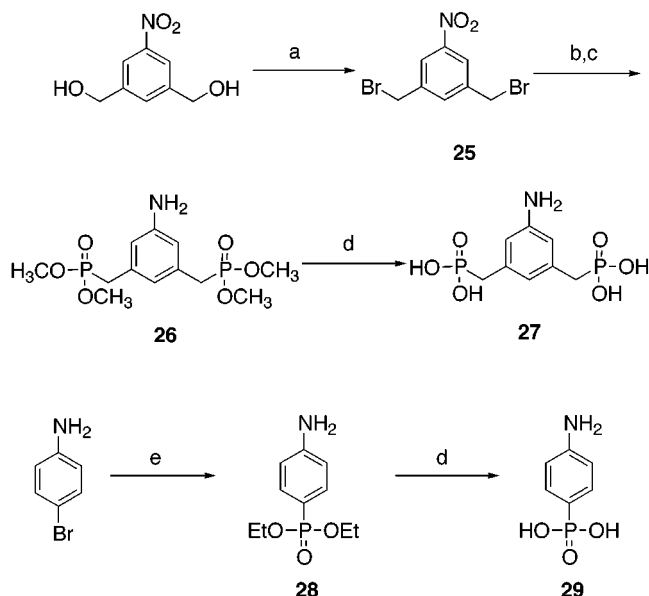
<sup>a</sup> System A was 0.1 M triethylammonium acetate buffer:CH<sub>3</sub>CN = 95:5 to 40:60 for 20 min with flow rate 1 mL/min. System B was 5 mM tetrabutylammonium phosphate buffer:CH<sub>3</sub>CN 80:20 to 40:60 in 20 min with flow rate 1 mL/min.

selectivity and high affinity can be achieved through structural modification of the pyridoxal-5'-phosphate series of antagonists. Recently we reported<sup>14</sup> that the cyclic pyridoxine- $\alpha^{4,5}$ -monophosphate corresponding to **2**, i.e. compound **3**, was a weak but selective antagonist of ATP-evoked responses at rat P2X<sub>1</sub> receptors. Furthermore, this cyclic phosphate was inactive at phospholipase C-coupled P2Y receptors, at the adenylate cyclase-coupled P2Y receptors in rat C6 glioma cells, and at adenosine receptors. In the present study, additional analogues of both pyridoxal and pyridoxine are explored, resulting in enhanced potency and/or selectivity at P2X<sub>1</sub> receptors. In certain analogues, the polyanionic nature of the derivatives is diminished, and in other analogues the stability is potentially enhanced through the introduction of a phosphonate linkage. As antagonists, many of the compounds were found to be more freely reversible, in comparison to **1** and **2**. Some of the present 5'-phosphate and 5'-phosphonate derivatives were reported in a preliminary study<sup>25</sup> and are now characterized more fully at a broader range of recombinant P2X receptor subtypes.

## Results

We have introduced 5'-phosphonate linkages,<sup>25</sup> cyclic phosphate diesters,<sup>14</sup> bisphosphates, and modified functional groups on the phenylazo moiety (Table 1), with the aim of developing more potent and selective antagonists for P2 receptor subtypes. Halo, carboxylate, sulfonate, and phosphonate (Scheme 1) variations were made on the phenyl ring (compounds **4**–**11**). 5-Methylphosphonate and 5-ethylphosphonate substitutions of the methylphosphate moiety were introduced. Ana-

## Scheme 1. Synthesis of Anilinephosphonic Acid Derivatives<sup>a</sup>



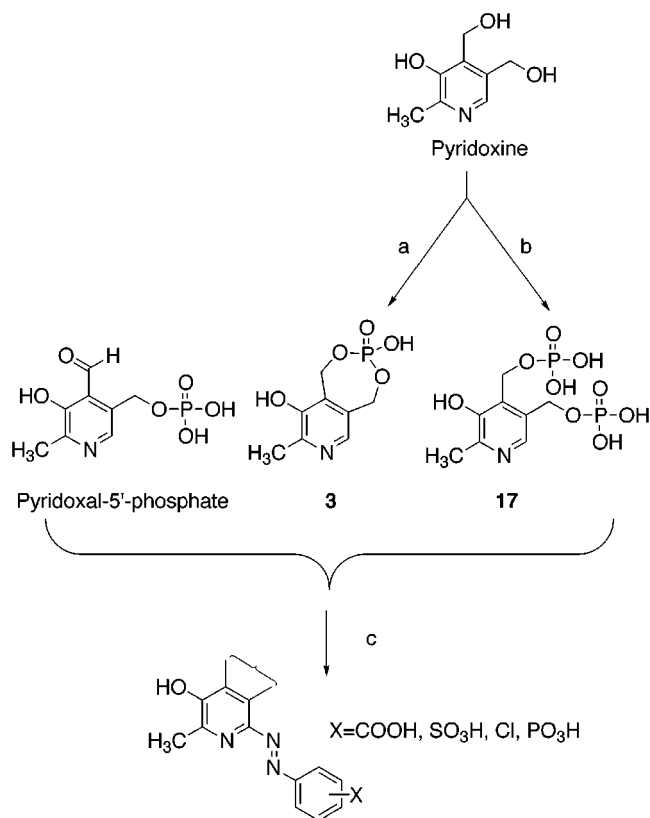
<sup>a</sup> Reagents: (a) (Ph)<sub>3</sub>P, CBr<sub>4</sub> in ether, 25 °C, 12 h, 52%; (b) P(OCH<sub>3</sub>)<sub>3</sub>, 80 °C, 6 h, 94%; (c) Pd/C, H<sub>2</sub> in MeOH, 1 atm, 25 °C, 1 h, 100%; (d) TMS-Br in CH<sub>3</sub>CN, 25 °C, 12 h, 99%; (e) (EtO)<sub>2</sub>P(O)H, triethylamine, tetrakis(Ph)<sub>3</sub>Pd(0) in toluene, 90 °C, 16 h, 18%.

logues of the P2 receptor antagonists pyridoxal-5'-phosphate and 6-azophenyl-2',5'-disulfonate derivative (**2**), in which the phosphate group was cyclized by esterification to a CH<sub>2</sub>OH group at the 4-position in pyridoxine, were synthesized (compounds **13**–**16**). Finally, bisphosphate derivatives of pyridoxine (compounds **18**–**20**) were prepared (Scheme 2).

The new derivatives were characterized using NMR and high-resolution mass spectroscopy, and purity of 95–98% was demonstrated using high-pressure liquid chromatography (HPLC) in two different solvent systems. The compounds were tested in ion channel assays<sup>26</sup> of ATP-induced current at recombinant rat P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptors, expressed in *Xenopus* oocytes, using the twin-electrode voltage-clamping technique.

Several compounds (**4**, **8**, **10**) were previously reported to antagonize agonist-induced cation flux at recombinant P2X<sub>2</sub> receptors, with IC<sub>50</sub> values of 1–10 μM. Most of the compounds were weak or inactive as antagonists at the turkey erythrocyte P2Y<sub>1</sub> receptor (Table 2), in activation of phospholipase C activity<sup>27,28</sup> induced by 10 nM 2-methylthioadenosine-5'-diphosphate (2-MeSADP). Compound **11** displayed an IC<sub>50</sub> value of 27 μM.

In inhibition of the inward current elicited by ATP at recombinant P2X<sub>1</sub> receptors, compounds **4**–**11**, with IC<sub>50</sub> values of 9–42 nM, were found to be more potent than **1** and **2** (IC<sub>50</sub> values of 99 and 43 nM, respectively). The *p*-carboxylate analogue, **4**, having a phosphate linkage similar to compound **1**, selectively inhibited ATP-evoked responses at P2X<sub>1</sub> receptors with an IC<sub>50</sub> value of 9 nM and displayed 1300-, 16-, and >10000-fold selectivity versus P2X<sub>2</sub>, P2X<sub>3</sub>, and P2Y<sub>1</sub> receptors, respectively. The corresponding 5-methylphosphonate analogue, **5**, was equipotent to **4** at P2X<sub>1</sub> receptors (Figure 2). Thus, compound **5** was 16-fold selective for P2X<sub>1</sub> versus P2X<sub>3</sub> receptors and 19-fold selective versus P2X<sub>2</sub> receptors. The monocarboxylic acid 5-ethylphosphonate analogue, **6**, and the dicarboxylic acid deriva-

**Scheme 2.** Azo Coupling Reaction of Pyridoxal- and Pyridoxine-Phosphate Derivatives<sup>a</sup>

<sup>a</sup> Reagents: TMS-polyphosphate, benzene, 2 days, either (a) 5% solution, 40 °C or (b) 20% solution, 85 °C; (c) aromatic amine, NaNO<sub>2</sub>, 6 N HCl, pH 10–11.

tive, **7**, were slightly less potent than **5** at P2X<sub>1</sub> receptors. Thus, the phosphonate substitution of the 5-methylphosphate moiety, anticipated to increase stability of the analogues to hydrolysis, was found to be well-tolerated at P2X receptor subtypes.

Phosphonate substitution of the arylazo ring proved to have distinctive effects on potency and selectivity. The IC<sub>50</sub> values for the bis(methylphosphonate), **9**, at P2X<sub>1</sub> and P2X<sub>3</sub> receptors were 11 and 25 nM, respectively, in the presence of 1 and 3 μM ATP, respectively, thus the P2X<sub>3</sub> receptor potency was enhanced. Compound **9** was approximately 25- and 1300-fold selective for the P2X<sub>1</sub> subtype versus P2X<sub>2</sub> and P2Y<sub>1</sub> receptors, respectively. Compound **10**, a 5-methylphosphonate having the same arylazo sulfonate substitution as compound **2**, antagonized responses at P2X<sub>1</sub> receptors with an IC<sub>50</sub> value of 12 nM. Compound **10** was 28-fold selective for P2X<sub>1</sub> versus P2X<sub>3</sub> receptors and 92-fold selective versus P2X<sub>2</sub> receptors. A *p*-phenylphosphonate derivative, **11** (Figure 3), was nearly as potent as **9** at both P2X<sub>1</sub> and P2X<sub>3</sub> receptors. Thus, **11** was 79-fold selective for P2X<sub>1</sub> versus P2X<sub>2</sub> receptors.

The time course of washout at P2X<sub>1</sub> and P2X<sub>3</sub> receptors of various potent antagonists was examined (Figure 4). Ion channel activity was more rapidly restored, following a 20-min washout, for several active compounds than for **1**. The percent of control activity recovered for **1** following washout was only 30–40%. The action of **2** was fully reversible at both receptors, and most other analogues (**4**, **8**–**11**), except for compounds **5** and **7** with 20–30%, displayed ≥75% recovery.

The cyclic phosphates and bisphosphates derived from pyridoxine (**3**, **13**–**20**) were much less potent than the pyridoxal derivatives in antagonizing the activation of recombinant P2X receptors expressed in oocytes. The cyclic phosphate analogue unsubstituted on the arylazo ring, **16**, was inactive.

Compound **15**, a cyclic phosphate in which the 3-OH group was acetylated, was completely inactive at P2X<sub>1</sub> receptors, which in comparison to **3** demonstrates the importance of the hydroxyl group for receptor recognition.

Selected derivatives of each major class of derivatized pyridoxal-phosphate were also tested for relative antagonistic actions on human recombinant P2X<sub>7</sub> receptors expressed in HEK (human embryonic kidney) 293 cells (Table 3). For comparison, three additional analogues of **2**, e.g. **21**–**23**, and a nucleotide antagonist of P2Y<sub>1</sub> receptors,<sup>28</sup> **24**, were examined in the P2X<sub>7</sub> receptor assay. Activation of this particular P2X family receptor induces two distinct changes in plasma membrane permeability: (1) a rapidly gated cation channel permeable to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> and (2) a delayed pore that is nonselectively permeable to both inorganic ions and various organic molecules up to 800 Da in mass. We first assayed the rapidly gated cation channel activity by assaying the ATP-induced loss of total cell K<sup>+</sup>. Given the strong inhibitory effects of extracellular divalent cations on P2X<sub>7</sub> receptor function,<sup>29</sup> these assays were performed using cells suspended in media containing physiological concentrations of Mg<sup>2+</sup> and Ca<sup>2+</sup> or in media containing no added Mg<sup>2+</sup> or Ca<sup>2+</sup>. In physiological saline containing 1.5 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, the EC<sub>50</sub> for ATP was 1200 μM (as assayed by K<sup>+</sup> efflux). Thus, we used 3 mM ATP as a maximally active concentration of agonist. Under these conditions, 30 μM **2** produced an 81 ± 10% inhibition (*n* = 3 experiments) of the K<sup>+</sup> release triggered by 3 mM ATP. None of the other derivatized pyridoxal-phosphate compounds matched the efficacy of **2**. Compound **10** was the next most efficacious inhibitor (51 ± 6% inhibition) followed by the related compound **24**. A similar rank order of efficacy was observed using HEK-hP2X<sub>7</sub> cells tested in medium lacking extracellular Mg<sup>2+</sup> and Ca<sup>2+</sup>. The relative efficacy of all inhibitory compounds, including **2**, was reduced in the absence of extracellular Mg<sup>2+</sup> and Ca<sup>2+</sup>.

We next assayed the effects of these compounds on ATP-induced pore formation by the P2X<sub>7</sub> receptor. Because pore formation is very strongly repressed by extracellular Mg<sup>2+</sup> and Ca<sup>2+</sup>, these experiments were performed using divalent cation-free saline. Under these assay conditions the EC<sub>50</sub> for ATP-induced activation of the pore was 120 μM. Thus, we used 300 μM ATP as a maximally active concentration of the agonist. 30 μM **2** completely repressed ATP-induced pore formation. Compound **10**, the phosphonate derivative of **2**, was equally efficacious (100% inhibition at 30 μM). Introduction of bis(methylphosphonate) moieties into the pyridoxal-phosphate core structure (compound **9**) further reduced the antagonistic action at the human P2X<sub>7</sub> receptor. Cyclic phosphate (compound **3**) and bisphosphate derivatives (compounds **18** and **19**) were inactive.

The same rank order of efficacy (**2** > **10** > **24** > **9**, **22**, **21** > **3**, **18**, **19**) characterized the inhibitory effects of



**Table 2.** Structures and Pharmacological Activities of Pyridoxal and Pyridoxine Derivatives (phosphates and phosphonates) at P2 Receptors

compd	positions					recombinant IC <sub>50</sub> (μM)			PLC assay IC <sub>50</sub> (μM)	
	2'	3'	4'	5'	X	P2X <sub>1</sub> <sup>a</sup> or % inhib	P2X <sub>2</sub> <sup>a</sup>	P2X <sub>3</sub> <sup>a</sup>	P2Y <sub>1</sub> <sup>c</sup>	
<b>1</b> (PPADS)	SO <sub>3</sub> H	H	SO <sub>3</sub> H	H	CH <sub>2</sub> O	0.099 ± 0.006	1.6 ± 0.1	0.240 ± 0.038	16.6 ± 2.5	
<b>2</b> (IsoPPADS)	SO <sub>3</sub> H	H	H	SO <sub>3</sub> H	CH <sub>2</sub> O	0.043 ± 0.018	0.398 ± 0.125	0.084 ± 0.004	21.4 ± 9.0	
<b>4</b> <sup>d,f</sup>	H	H	COOH	H	CH <sub>2</sub> O	0.0094 ± 0.0017	11.9 ± 1.4	0.140 ± 0.011	~100	
<b>5</b>	H	H	COOH	H	CH <sub>2</sub>	0.0081 ± 0.0021	0.150 ± 0.020	0.128 ± 0.019		
<b>6</b> <sup>d</sup>	H	H	COOH	H	CH <sub>2</sub> CH <sub>2</sub>	0.020 ± 0.003	2.4 ± 0.3	0.145 ± 0.057	~100	
<b>7</b>	COOH	H	H	COOH	CH <sub>2</sub>	0.037 ± 0.008	0.486 ± 0.023	0.330 ± 0.014		
<b>8</b> <sup>d</sup>	H	H	H	H	CH <sub>2</sub> O	0.042 ± 0.006	1.2 ± 0.2	0.480 ± 0.090	54 ± 3	
<b>9</b> <sup>f</sup>	H	CH <sub>2</sub> P(O)(OH) <sub>2</sub>	H	CH <sub>2</sub> P(O)(OH) <sub>2</sub>	CH <sub>2</sub>	0.011 ± 0.005	0.280 ± 0.030	0.025 ± 0.007	14.5 ± 2.1	
<b>10</b> <sup>d,f</sup>	SO <sub>3</sub> H	H	H	SO <sub>3</sub> H	CH <sub>2</sub>	0.012 ± 0.003	1.1 ± 0.2	0.340 ± 0.040	46 ± 21	
<b>11</b> <sup>f</sup>	H	H	P(O)(OH) <sub>2</sub>	H	CH <sub>2</sub>	0.012 ± 0.008	0.948 ± 0.019	0.036 ± 0.010	27 ± 3	
<b>12</b> <sup>e</sup>						5.9 ± 1.8 (EC <sub>50</sub> ) <sup>g</sup>	inactive	inactive	inactive	
<b>13</b>	SO <sub>3</sub> H	H	H	SO <sub>3</sub> H	H	10.2 ± 2.6	inactive	58.3 ± 0.1	inactive	
<b>14</b>	Cl	H	H	SO <sub>3</sub> H	H	43 ± 4% <sup>b</sup>				
<b>15</b>	H	H	COOH	H	H	39 ± 10% <sup>b</sup>				
<b>16</b>	SO <sub>3</sub> H	H	H	SO <sub>3</sub> H	CH <sub>3</sub> CO	inactive				
<b>17</b>	H	H	H	H	H	inactive				inactive
<b>18</b>	SO <sub>3</sub> H	H	H	SO <sub>3</sub> H		27 ± 7% <sup>b</sup>			~100	
<b>19</b>	Cl	H	H	SO <sub>3</sub> H		78 ± 7% <sup>b</sup>		30 ± 10% <sup>b</sup>	~100	
<b>20</b>	H	H	H	H		50 ± 4% <sup>b</sup>			>100	

<sup>a</sup> Inhibition of ion current (mean ± SEM, *n* = 4), unless noted, induced by ATP (at the EC<sub>70</sub> values in μM for respective subtypes: P2X<sub>1</sub> 1, P2X<sub>2</sub> 10, and P2X<sub>3</sub> 3) at recombinant P2X receptors expressed in *Xenopus* oocytes (<sup>b</sup>% inhibition at 10 μM). <sup>c</sup> Inhibition of 10 nM 2-MeSADP-stimulated phospholipase C in turkey erythrocyte membranes (mean ± SEM, *n* = 3), labeled using [<sup>3</sup>H]inositol. <sup>d</sup> Compounds reported by Kim et al.<sup>25</sup> <sup>e</sup> Compounds and values reported by Jacobson et al.<sup>14</sup> <sup>f</sup> Compound **4**, MRS 2159; compound **5**, MRS 2284; compound **9**, MRS 2257; compound **10**, MRS 2191; compound **11**, MRS 2273. <sup>g</sup> Compound **12** potentiates the effect of ATP at P2X receptors.<sup>14</sup>

all tested compounds on both indices of human P2X<sub>7</sub> receptor activity. The apparent efficacies of the three most effective compounds (**2**, **9**, **10**) as human P2X<sub>7</sub> antagonists were significantly reduced in the presence of physiological divalent cation concentrations. In addition, these three compounds were significantly less potent and efficacious antagonists of the murine P2X<sub>7</sub> receptor even when assayed in the absence of divalent cations (data not shown). Previous studies have noted significant differences in the pharmacological antagonism of human versus rodent P2X<sub>7</sub> receptors.<sup>29</sup>

## Discussion

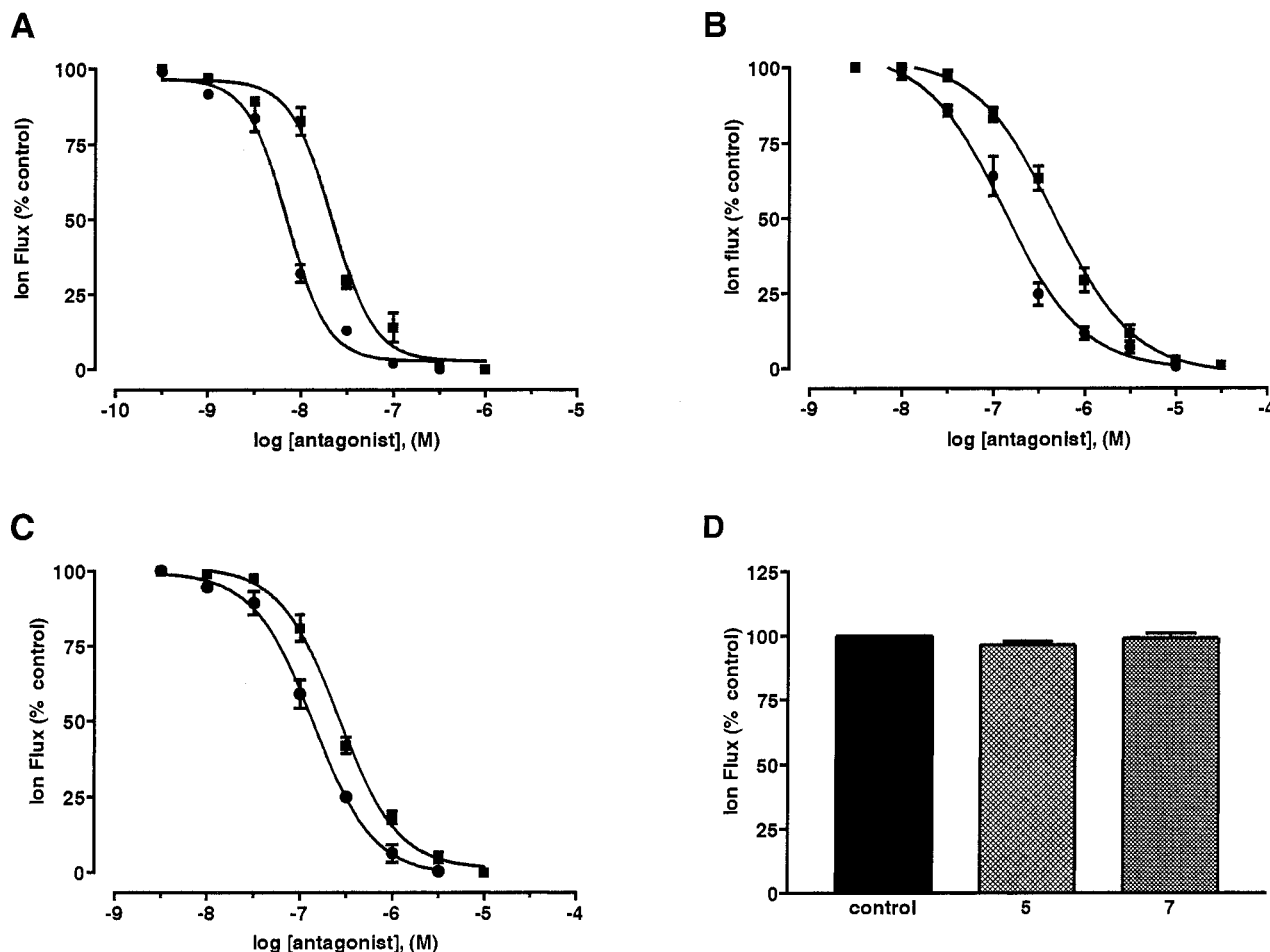
Compound **1** and its analogues have been studied at a variety of P2 receptor subtypes. At P2Y<sub>1</sub> receptors, a range of potencies of **1** has been reported, from approximately 1 μM<sup>30</sup> to 16.6 μM in the present study. Boarder and colleagues<sup>31</sup> also showed that **1** had a curious potentiating effect at turkey P2Y<sub>1</sub> receptors, which may be based on the inhibition of locally released ATP by a mechanosensory mechanism (stimulated by washing cells). Nevertheless, the potency of analogues of **2** prepared in the present study generally tended to be greater at P2X<sub>1-3</sub> subtypes than at P2Y<sub>1</sub> receptors. Furthermore, among the present set of analogues in which the aldehyde group remained, potencies often increased over **2** at the P2X<sub>1</sub> and P2X<sub>3</sub> subtypes and

decreased at the P2X<sub>7</sub> receptor.

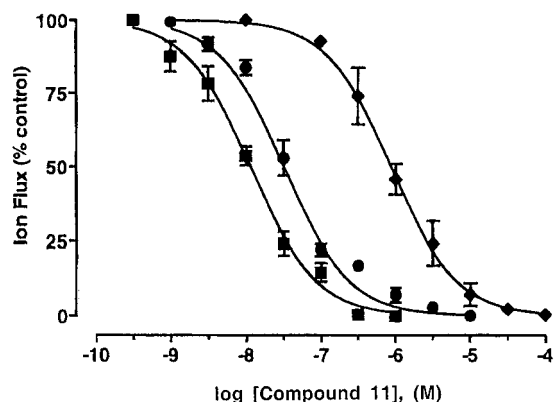
Functional group variation on the 6-(phenylazo) substituent was found to greatly modulate the potency as P2 receptor antagonists. For example, two analogues bearing phosphonate groups on the arylazo ring, i.e. compounds **9** and **11**, were selective for "group 1" (P2X<sub>1</sub> and P2X<sub>3</sub> receptors) versus "groups 2–4" (P2X<sub>2,4,7</sub>) receptors.

The view that **1** is an irreversible antagonist stemmed originally from pharmacokinetic data on blockade of P2X receptors in whole tissues. Compound **1** was noted as having a slow on-rate (slow equilibration) and slow off-rate (slow reversibility) at native P2X receptors in vascular and visceral smooth muscle (for a review, see ref 13). The fact that antagonism does eventually reverse resulted in the term "pseudo-irreversible" to describe the slow kinetics of these blocking agents.<sup>32</sup> Nonetheless, problems associated with diffusion, surface adhesion, uptake, compartmentalization, and re-release of **1** have never been clearly resolved where whole tissue assays are employed to test their activity. Most of these problems are avoided if recombinant P2X receptors are used instead, since the antagonist has direct access to the cell surface and receptor and drug concentration can be controlled by fast application and washout.

The slow onset and slow reversal of blockade by **1** at the recombinant P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptors (rat)



**Figure 2.** Potent effects of compounds **5** (●) and **7** (■) on inward current induced by ATP at recombinant rat P2X<sub>1</sub> (A) receptors and intermediate potency at rat P2X<sub>2</sub> (B) and rat P2X<sub>3</sub> (C) receptors. Compounds **5** and **7** (10  $\mu$ M) were inactive at rat P2X<sub>4</sub> (D) receptors. Receptors were expressed in *Xenopus* oocytes and current was measured using the twin-electrode voltage-clamping technique (pH 7.5, Ba<sup>2+</sup> Ringer's solution). All data points were mean  $\pm$  SEM of 4 observations. ATP concentrations were as in Materials and Methods. IC<sub>50</sub> values are given in Table 2. Hill coefficients ( $n_H$ ) were: (A)  $-1.20 \pm 0.07$  (**5**) and  $-1.19 \pm 0.08$  (**7**); (B)  $-1.11 \pm 0.009$  (**5**) and  $-0.95 \pm 0.15$  (**7**); (C)  $-0.87 \pm 0.04$  (**5**) and  $-0.89 \pm 0.05$  (**7**).

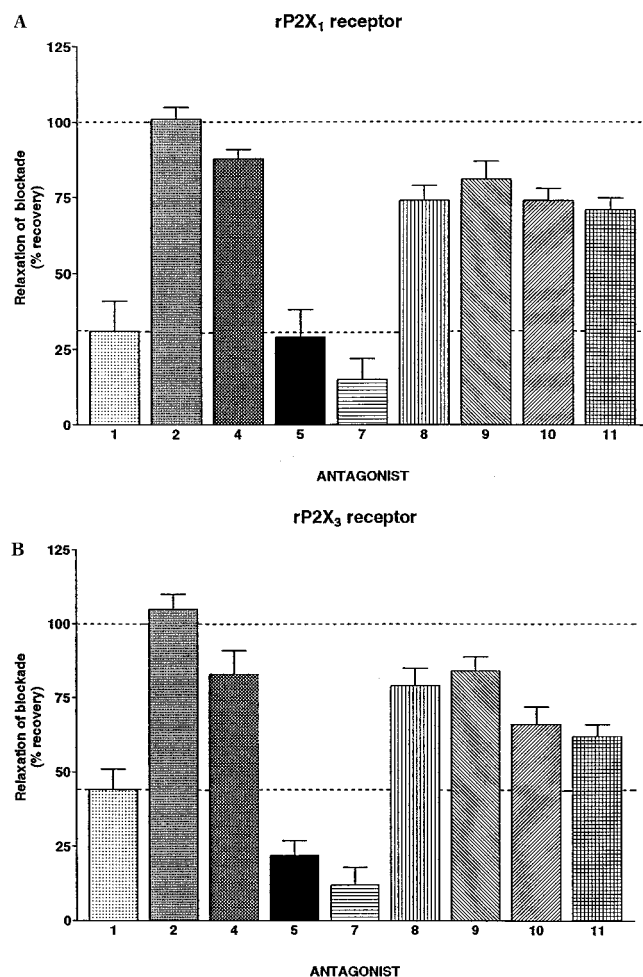


**Figure 3.** Selectivity of compound **11** on inward current induced by ATP at recombinant rat P2X<sub>1</sub> (■) and rat P2X<sub>3</sub> (●) receptors ("group 1") versus rat P2X<sub>2</sub> (◆) receptors ("group 2"), expressed in *Xenopus* oocytes, using the twin-electrode voltage-clamping technique (pH 7.5, Ba<sup>2+</sup> Ringer's solution). All data points were mean  $\pm$  SEM of 4 observations. ATP concentrations were as in Materials and Methods. IC<sub>50</sub> values are given in Table 2. Hill coefficients ( $n_H$ ) were: (P2X<sub>1</sub>)  $-1.05 \pm 0.05$ , (P2X<sub>2</sub>)  $-1.11 \pm 0.06$ , and (P2X<sub>3</sub>)  $-1.03 \pm 0.04$ .

led to the suggestion that the aldehyde group on the pyridoxal moiety formed a Schiff's base with a strategic lysyl residue at equivalent extracellular positions in P2X

subunits (K249 on rP2X<sub>1</sub>, K246 on rP2X<sub>2</sub>, K251 on rP2X<sub>3</sub>).<sup>33</sup> However, evidence for this assumption is far from clear. Point mutation of the lysine in the rP2X<sub>2</sub> subunit (K246E) did increase the rate of recovery from blockade by **1**, but it did not significantly affect the potency of the antagonist.<sup>33</sup> On the other hand, substitution of glutamate for lysine at the rat P2X<sub>4</sub> subunit (E249K) increased the potency of **1** (IC<sub>50</sub> = >500  $\mu$ M (wt) and 2.6  $\mu$ M (mutant)) yet did not noticeably accelerate recovery from blockade.<sup>33</sup> A similar introduction of lysine in the P2X<sub>6</sub> subunit (L251K) increased the potency of **1** (IC<sub>50</sub> = >500  $\mu$ M (wt) and 2.0  $\mu$ M (mutant)) without affecting the rate of recovery.<sup>34</sup>

Human and mouse forms of P2X<sub>4</sub> do not possess a lysine residue at position 249 yet are significantly more sensitive to **1** (27.5 and 21  $\mu$ M, respectively) than rP2X<sub>4</sub>.<sup>35,36</sup> Although sensitive to the antagonist, human and mouse P2X<sub>4</sub> receptors also recover very slowly from blockade by **1**. Mutation of rP2X<sub>4</sub> (N127K) to provide a lysine unique to hP2X<sub>4</sub> did not enhance the sensitivity of the rat orthologue to **1**.<sup>35</sup> Furthermore, mP2X<sub>4</sub> only possesses extracellular lysyl residues that are common to rP2X<sub>4</sub>,<sup>36</sup> and consequently, the heightened sensitivity to **1** of the former cannot be ascribed to a strategic lysine. The P2X<sub>3</sub> subunit lacks a lysine residue at the



**Figure 4.** Histogram showing the degree of recovery rat P2X<sub>1</sub> (A) and rat P2X<sub>3</sub> (B) receptors from full blockade by various antagonists, 20 min after drug washout. Compounds are indicated by their appropriate number, as shown in Table 2.

equivalent position, at which a threonine residue is found (T235), and yet **1** is potent ( $IC_{50} \sim 1 \mu M$ ) and recovery from blockade is rapid for both rat and human orthologues.<sup>35,37</sup> Thus, the critical role of a strategic lysyl residue in condensation with the aldehyde of **1** is difficult to reconcile given the available data.

In the present study, the  $IC_{50}$  value for **1** at the rP2X<sub>1</sub> receptor was lower than at the P2X<sub>3</sub> receptor (0.099 versus 0.240  $\mu M$ ; see Table 2), and the rate of recovery was faster at the rP2X<sub>3</sub> receptor. Compound **2** proved to be more potent at both rP2X<sub>1</sub> and rP2X<sub>3</sub> receptors, yet full recovery occurred at these receptors within 20 min of drug washout. Blockade by **2** at the P2X<sub>1</sub>-like receptor in rat vagus nerve was considered surmountable and a slow recovery reported.<sup>38</sup> Similarly, blockade by **2** was reversible at P2X receptors of rat vagus nerve bundle,<sup>22</sup> in which P2X<sub>3</sub> mRNA is present. Furthermore, it was shown that reversal of blockade by **1** at human P2X<sub>3</sub> receptors is accelerated by Cibacron blue which, of itself, potentiates ATP responses at this P2X receptor.<sup>40</sup> Some of the derivatives of **1** tested in the present study (e.g. compounds **4**, **8–11**) showed a level of recovery of about 75% and greater at rP2X<sub>1</sub> and rP2X<sub>3</sub> receptors within 20 min of washout. Thus, it now seems unnecessary to alter the aldehyde on the pyridoxal moiety, as with a pyridoxine cyclic phosphate, **3**,<sup>14</sup> to make allowances for Schiff's base formation and obtain

**Table 3.** Antagonistic Effects of Pyridoxal-Phosphate Derivatives on the Function of Human P2X<sub>7</sub> Receptors Expressed in HEK Cells: Cation Channel Activity versus Nonselective Pore

compd	% inhibition of ATP-induced		
	K <sup>+</sup> efflux (with cations) <sup>a</sup>	K <sup>+</sup> efflux (cation-free) <sup>b</sup>	ethidium influx <sup>c</sup>
<b>2</b>	81 ± 10	67 ± 14	100 ± 0
<b>9</b>	0 ± 3	-5 ± 3	84 ± 10
<b>10</b>	51 ± 6	28 ± 7	100 ± 0
<b>3</b>	-1 ± 3	-5 ± 2	17 ± 15
<b>18</b>	-1 ± 6	-7 ± 4	15 ± 15
<b>19</b>	-1 ± 5	-5 ± 3	12 ± 15
<b>21</b> <sup>d</sup>	14 ± 2	2 ± 2	92 ± 15
<b>22</b> <sup>d</sup>	4 ± 4	1 ± 2	96 ± 7
<b>23</b> <sup>d</sup>	-5 ± 6	-8 ± 1	40 ± 35
<b>24</b> <sup>d</sup>	37 ± 1	24 ± 10	100 ± 0

<sup>a</sup> Adherent HEK293 cells stably expressing the recombinant human P2X<sub>7</sub> receptor; antagonist, 30  $\mu M$ ; agonist, 3 mM ATP in divalent cation-containing medium;  $n = 3$ . <sup>b</sup> Adherent HEK293 cells stably expressing the recombinant human P2X<sub>7</sub> receptor; antagonist, 30  $\mu M$ ; agonist, 3 mM ATP in divalent cation-free medium;  $n = 3$ . <sup>c</sup> Suspended HEK293 cells stably expressing the recombinant human P2X<sub>7</sub> receptor; antagonist, 30  $\mu M$ ; agonist, 300  $\mu M$  ATP in divalent cation-containing medium;  $n = 3$ . <sup>d</sup> **21**, MRS 2157 = pyridoxal- $\alpha^5$ -phosphate-6-azophenyl-2'-chloro-5'-sulfonic acid; **22**, MRS 2160 = pyridoxal- $\alpha^5$ -phosphate-6-azophenyl-3'-chloro-4'-carboxylic acid; **23**, MRS 2192 = [4-formyl-3-hydroxy-2-methyl-6-(2-chloro-5-sulfonylphenylazo)pyrid-5-yl]methylphosphonic acid;<sup>25</sup> **24**, MRS 2179 = 2'-deoxy-N<sup>6</sup>-methyladenosine-3',5'-bisphosphate.<sup>28</sup>

reversible antagonism at these P2X receptors. Recently, a naphthyl derivative of **1** that also retains the aldehyde group, pyridoxal- $\alpha^5$ -phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonic acid), was shown to be a potent selective and reversible antagonist of P2X<sub>1</sub> receptors.<sup>39</sup>

Some P2 receptor antagonists also may inhibit ectonucleotidases, making these studies even more complex. We are currently studying ectonucleotidase inhibition by selected analogues of **2**.<sup>41</sup> Preliminary results with the recombinant enzymes expressed in CHO cells indicate that most of the analogues of **1** were better inhibitors of ecto-apyrase than of ecto-ATPase. The aldehyde group appears to be required for ecto-apyrase inhibition. However, the efficacy of derivatives of **1** as inhibitors of ecto-enzymes had no bearing on their efficacy as antagonists at P2X<sub>1–3</sub> receptors expressed in *Xenopus* oocytes, since defolliculated oocytes are largely devoid of surface enzymes that degrade ATP.<sup>42</sup>

In conclusion, we have identified highly potent and selective antagonists of P2X<sub>1</sub> receptors and antagonists of combined P2X<sub>1,3</sub> receptor selectivity. Damer et al.<sup>43</sup> have also reported an antagonist with high affinity and apparent P2X<sub>1</sub> receptor selectivity: the suramin analogue NF279 (8,8'-(carbonylbis(imino-4,1-phenylene-carbonylimino-4,1-phenylene-carbonylimino))bis(1,3,5-naphthalenetrisulfonic acid)). A selective P2X<sub>1</sub> receptor antagonist may have potential utility in controlling receptor-mediated contraction of visceral and vascular smooth muscle (e.g. vascular hypertension and instability of the urinary bladder detrusor muscle). A selective P2X<sub>3</sub> receptor antagonist may be useful in pain control. The properties of selective P2 receptor antagonists in the central nervous system remain to be examined.

## Materials and Methods

**Synthesis. Materials and Analytical Methods.** Compounds **4**, **6**, **8**, **10** and (4-formyl-3-hydroxy-2-methyl-5-pyridyl)-



methylphosphonic acid, as the starting compound for the synthesis of compounds **5**, **7**, and **9–11**, were prepared as described in a previous report.<sup>25</sup> Compounds **3** and **12** were synthesized according to the literature.<sup>14</sup> Pyridoxine and the reagents for azo coupling reactions were purchased from Aldrich (St. Louis, MO). Aniline-2,5-disulfonic acid was obtained from K & K Laboratories, Inc. (Hollywood, CA).

<sup>1</sup>H NMR spectroscopy was performed on a Varian GEMINI-300 spectrometer and spectra were taken in D<sub>2</sub>O or CDCl<sub>3</sub>. The chemical shifts are expressed as relative ppm from HOD peaks (4.78 ppm) or as ppm downfield from tetramethylsilane. <sup>31</sup>P NMR spectra were recorded without proton decoupling mode, at room temperature using a Varian XL-300 spectrometer (121.419 MHz); orthophosphoric acid (85%) was used as an external standard. High-resolution mass spectroscopy (HRMS) using FAB (fast atom bombardment) or EI (electron impact) was determined with a JEOL SX102 spectrometer, and electron spray mass spectra were obtained using a Hewlett-Packard 1100 LC-ESPRAY system.

The determinations of purity were performed with a Hewlett-Packard 1090 HPLC system using an OD-5-60 C18 analytical column (250 mm × 4.6 mm; Separation Methods Technologies, Inc., Newark, DE) in two different linear gradient solvent systems. One solvent system (A) was 0.1 M triethylammonium acetate buffer:CH<sub>3</sub>CN = 95:5 to 40:60 for 20 min with flow rate = 1 mL/min. The other (B) was 5 mM tetrabutylammonium phosphate buffer:CH<sub>3</sub>CN = 80:20 to 40:60 in 20 min with flow rate = 1 mL/min. Peaks were detected by UV absorption using a diode array detector.

**General Procedure A for Azo Coupling Reactions.** To a stirred solution of an aniline analogue (0.11 mmol) and 13 mg (0.12 mmol) of Na<sub>2</sub>CO<sub>3</sub> in 1 mL of H<sub>2</sub>O was added 8 mg (0.12 mmol) of NaNO<sub>2</sub> at 0 °C. 60 μL (0.36 mmol) of 6 N HCl was added dropwise, and the mixture was stirred for 5–10 min at 0 °C. A solution of a pyridoxal- or pyridoxine-phosphate analogue (0.1 mmol) in 1 mL of H<sub>2</sub>O was added at once, and the pH of the mixture was adjusted to 10–11 by addition of 1 N NaOH (~300 μL). The yellow color changed to red (sometimes gradually) as an indicator of the reaction occurring. After stirring for 30 min at 0 °C and 30 min at 25 °C, the mixture was purified by ion-exchange column chromatography using Amberlite CG-50 resin (H<sup>+</sup> form, weakly acidic) with the elution of water (flow rate 0.5 mL/min). The red fraction showing a single peak in HPLC was collected and lyophilized to give the desired compound with more than 95% purity.

**[4-Formyl-3-hydroxy-2-methyl-6-(4'-carboxyphenylazo)pyrid-5-yl]methylphosphonic Acid Disodium Salt (5).** Following the general procedure A with 23 mg (0.1 mmol) of (4-formyl-3-hydroxy-2-methyl-5-pyridyl)methylphosphonic acid and 16 mg (0.12 mmol) of 4-aminobenzoic acid, 27 mg of **5** was obtained (yield 71%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.41 (3H, s, -CH<sub>3</sub>), 3.85 (2H, d, *J* = 21.5 Hz, -CH<sub>2</sub>P-), 7.70 (2H, d, *J* = 7.8 Hz, phenyl), 7.88 (2H, d, *J* = 7.8 Hz, phenyl), 10.24 (1H, s, -CHO); <sup>31</sup>P NMR (D<sub>2</sub>O) δ -4.61 (t, *J* = 21.4 Hz).

**[4-Formyl-3-hydroxy-2-methyl-6-(2',5'-dicarboxyphenylazo)pyrid-5-yl]methylphosphonic Acid Disodium Salt (7).** Following the general procedure A with 23 mg (0.115 mmol) of (4-formyl-3-hydroxy-2-methyl-5-pyridyl)methylphosphonic acid and 22 mg (0.12 mmol) of 2-aminoterephthalic acid, 25 mg of **7** was obtained (yield 59%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.01 (3H, s, -CH<sub>3</sub>), 3.77 (2H, d, *J* = 21.5 Hz, -CH<sub>2</sub>P-), 7.48 (1H, d, *J* = 7.8 Hz, phenyl), 7.74 (1H, d, *J* = 7.8 Hz, phenyl), 8.01 (1H, s, phenyl), 10.09 (1H, s, -CHO); <sup>31</sup>P NMR (D<sub>2</sub>O) δ -4.31 (t, *J* = 21.5 Hz).

**[4-Formyl-3-hydroxy-2-methyl-6-azo(3',5'-bismethylphosphonylphenyl)-5-pyridyl]methylphosphonic Acid Disodium Salt (9).** Following the general procedure A with 46 mg (0.2 mmol) of (4-formyl-3-hydroxy-2-methyl-5-pyridyl)methylphosphonic acid and 56 mg (0.2 mmol) of 1-amino-3,5-bis(methylphosphonic acid), 24 mg of **9** was obtained (yield 21%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.54 (3H, s, -CH<sub>3</sub>), 3.19 (4H, d, *J* = 21.5 Hz, 2 × -CH<sub>2</sub>P-), 4.05 (2H, d, *J* = 21.5 Hz, -CH<sub>2</sub>P-), 7.39 (1H, s, phenyl), 7.76 (2H, s, phenyl), 10.36

(1H, s, -CHO); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 17.89 (t, *J* = 20.9 Hz), 21.69 (m), 22.29 (m).

**[4-Formyl-3-hydroxy-2-methyl-6-(4'-phosphonylphenylazo)pyrid-5-yl]methylphosphonic Acid Disodium Salt (11).** Following the general procedure A with 23 mg (0.115 mmol) of (4-formyl-3-hydroxy-2-methyl-5-pyridyl)methylphosphonic acid and 20 mg (0.115 mmol) of **29**, 36 mg of **11** was obtained (yield 68%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.48 (3H, s, -CH<sub>3</sub>), 3.99 (2H, d, *J* = 21.5 Hz, -CH<sub>2</sub>P-), 7.82–7.89 (4H, m, phenyl), 10.27 (1H, s, -CHO); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 12.83 (m), 17.89 (t, *J* = 21.4 Hz).

**Pyridoxine-α<sup>4,5</sup>-cyclomonophosphate-6-azophenyl-2'-chloro-5'-sulfonic Acid Monosodium Salt (13).** Following the general procedure A with 0.1 g (0.432 mmol) of **12** and 0.11 g (0.444 mmol) of aniline-2-chloro-5-sulfonic acid, 0.12 g of **5** was obtained (yield 59%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.47 (3H, s, -CH<sub>3</sub>), 5.18 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 5.61 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 7.56 (1H, d, *J* = 8.8 Hz, phenyl), 7.67 (1H, d, *J* = 8.8 Hz, phenyl), 7.95 (1H, s, phenyl); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 5.48 (pen, *J* = 15.9 Hz).

**Pyridoxine-α<sup>4,5</sup>-cyclomonophosphate-6-azophenyl-4'-carboxylic Acid (14).** Following the general procedure A with 50 mg (0.216 mmol) of **12** and 0.03 g (0.216 mmol) of 4-aminobenzoic acid, 35 mg of **14** was obtained (yield 43%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.32 (3H, s, -CH<sub>3</sub>), 5.00 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 5.47 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 7.58 (2H, d, *J* = 7.8 Hz, phenyl), 7.81 (2H, d, *J* = 8.8 Hz, phenyl); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 5.50 (pen, *J* = 15.9 Hz).

**O<sup>3</sup>-Acetylpyridoxine-α<sup>4,5</sup>-cyclomonophosphate-6-azophenyl-2',5'-disulfonic Acid Monotriethylamine Disodium Salt (15).** A mixture of 23 mg (0.043 mmol) of **3**, 100 μL of acetic anhydride and 100 μL of triethylamine in 1 mL of DMF was stirred for 1 h. The reaction mixture was purified with the same ion-exchange column chromatography in general procedure A to give 27 mg of **15** (100% yield): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 1.23 (9H, t, *J* = 6.8 Hz, 3 × -CH<sub>3</sub>), 2.42 (3H, s, -CH<sub>3</sub>), 2.49 (3H, s, -CH<sub>3</sub>), 3.15 (6H, q, *J* = 6.8 Hz, 3 × -CH<sub>2</sub>), 5.18 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 5.71 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 7.91 (1H, s, phenyl), 8.05 (1H, d, *J* = 7.8 Hz, phenyl), 8.18 (1H, d, *J* = 7.8 Hz, phenyl); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 5.08 (pen, *J* = 15.9 Hz).

**Pyridoxine-6-azophenyl-α<sup>4,5</sup>-cyclomonophosphoric Acid Monosodium Salt (16).** Following the general procedure A with 25 mg (0.108 mmol) of **12** and 24 mg (0.189 mmol) of aniline hydrochloride, 10 mg of **16** was obtained (yield 28%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.34 (3H, s, -CH<sub>3</sub>), 5.05 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 5.49 (2H, d, *J* = 15.6 Hz, -CH<sub>2</sub>O-), 7.37–7.39 (3H, m, phenyl), 7.59–7.62 (2H, m, phenyl); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 5.30 (pen, *J* = 15.9 Hz); HRMS (FAB-) calcd 334.0593, found 334.0607. HPLC retention time 7.2 min (purity >98%).

**Pyridoxine-α<sup>4,5</sup>-bisphosphate Tetraammonium Salt (17).** To a suspension of 0.5 g (2.96 mmol) of pyridoxine in 2.5 mL of anhydrous benzene was added 5 mL of trimethylsilyl polyphosphate under nitrogen atmosphere. The mixture was stirred at 85 °C for 2 days and poured into 30 mL of anhydrous ether. The white precipitate was collected by filtration, washed with anhydrous ether, and dissolved in 30 mL of water. The water solution was stirred at 70 °C for 12 h to cleave multiphosphate groups and remove trimethylsilyl group, and passed through Amberlite CG-50 resin (H<sup>+</sup> form, weakly acidic) with the elution of water (flow rate 0.5 mL/min). The acidic fractions with UV absorption were combined, lyophilized, redissolved 10 mL of water, and neutralized by triethylamine (pH ~7). The desired product was purified using Sephadex-DEAE A-25 (HCO<sub>3</sub><sup>-</sup> form) ion-exchange column chromatography with a linear gradient elution of water and 0.5 M ammonium bicarbonate solution. The pure fractions determined by HPLC were collected and repeated lyophilization with water gave 0.215 g of **17** (yield 18%): <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.43 (3H, s, -CH<sub>3</sub>), 4.92 (2H, s, -CH<sub>2</sub>O-), 4.94 (2H, d, *J* = 2.9 Hz, -CH<sub>2</sub>O-), 7.86 (1H, s, H-6); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 3.51 (t, *J* = 5.5 Hz), 4.40 (pt).

**Pyridoxine-α<sup>4,5</sup>-bisphosphate-6-azophenyl-2',5'-disulfonic Acid Disodium Salt (18).** Following the general



procedure A with 25 mg (0.063 mmol) of **17** and 16 mg (0.063 mmol) of aniline-2,5-disulfonic acid, 32 mg of **18** was obtained (yield 80%):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  2.72 (3H, s,  $-\text{CH}_3$ ), 5.32 (2H, d,  $J = 8.8$  Hz,  $-\text{CH}_2\text{O}-$ ), 5.68 (2H, d,  $J = 6.8$  Hz,  $-\text{CH}_2\text{O}-$ ), 8.04 (1H, d,  $J = 7.8$  Hz, phenyl), 8.13 (1H, s, phenyl), 8.18 (1H, d,  $J = 7.8$  Hz, phenyl);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  -0.06 (t,  $J = 6.8$  Hz), 1.67 (pt).

**Pyridoxine- $\alpha^{4,5}$ -bisphosphate-6-azophenyl-2'-chloro-5'-sulfonic Acid Monosodium Salt (19).** Following the general procedure A with 25 mg (0.063 mmol) of **17** and 16 mg (0.063 mmol) of aniline-2-chloro-5-sulfonic acid, 26 mg of **19** was obtained (yield 73%):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  2.68 (3H, s,  $-\text{CH}_3$ ), 5.31 (2H, d,  $J = 8.8$  Hz,  $-\text{CH}_2\text{O}-$ ), 5.67 (2H, d,  $J = 6.8$  Hz,  $-\text{CH}_2\text{O}-$ ), 7.79 (1H, d,  $J = 7.8$  Hz, phenyl), 7.92 (1H, dd,  $J = 2.0, 7.8$  Hz, phenyl), 8.18 (1H, d,  $J = 2.0$  Hz, phenyl);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  -0.06 (t,  $J = 6.2$  Hz), 1.69 (pt).

**Pyridoxine-6-azophenyl- $\alpha^{4,5}$ -bisphosphoric Acid Monosodium Salt (20).** Following the general procedure A with 25 mg (0.063 mmol) of **17** and 24 mg (0.189 mmol) of aniline hydrochloride, 22 mg of **20** was obtained (yield 81%):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  2.54 (3H, s,  $-\text{CH}_3$ ), 5.20 (2H, d,  $J = 7.8$  Hz,  $-\text{CH}_2\text{O}-$ ), 5.55 (2H, d,  $J = 4.9$  Hz,  $-\text{CH}_2\text{O}-$ ), 7.57–7.59 (3H, m, phenyl), 7.93–7.96 (2H, m, phenyl);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  0.08 (t,  $J = 5.2$  Hz), 1.54 (t,  $J = 7.9$  Hz).

**3,5-Bis(bromomethyl)-1-nitrobenzene (25).** A solution of 1.89 g of 5-nitro-*m*-xylene-( $\alpha,\alpha'$ )-diol (10 mmol), 5.25 g of triphenylphosphine (20 mmol), and 6.62 g of carbon tetrabromide (20 mmol) in 50 mL of anhydrous ether was stirred at 25 °C for 12 h under  $\text{N}_2$  atmosphere. After evaporation, the residue was purified with flash silica gel column chromatography (Hex/EtOAc = 5/1) to give 1.6 g of **25** as a bright yellow solid (52%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.53 (4H, s,  $2 \times -\text{CH}_2\text{Br}$ ), 6.83 (1H, s, Ph), 7.76 (1H, s, Ph), 8.20 (1H, s, Ph); MS (EI) ( $\text{M}^+$ ) 309; HRMS (EI) calcd 306.8843, found 306.8859.

**Tetramethyl 1-Aminobenzene-3,5-bis(methylphosphonate) (26).** A solution of 1.4 g of **25** (4.53 mmol) in 15 mL of trimethyl phosphite was heated at 80 °C for 6 h. After evaporation, the residue was purified with flash silica gel column chromatography ( $\text{CHCl}_3/\text{MeOH} = 30/1$ ) to give 1.57 g of tetramethyl 1-nitrobenzene-3,5-bis(methylphosphonate) as a white solid (94%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.25 (4H, d,  $J = 21.5$  Hz,  $2 \times -\text{CH}_2\text{P}-$ ), 3.72 (3H, s,  $-\text{CH}_3$ ), 3.73 (3H, s,  $-\text{CH}_3$ ), 3.75 (3H, s,  $-\text{CH}_3$ ), 3.76 (3H, s,  $-\text{CH}_3$ ), 7.28 (1H, s, Ph), 7.60 (1H, s, Ph), 8.06 (1H, s, Ph);  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  27.09 (m), 33.48 (m); MS (EI) ( $\text{M}^+$ ) 367 HRMS (EI) calcd 367.0586, found 367.0592.

A solution of 40 mg of tetramethyl 1-nitrobenzene-3,5-bis(methylphosphonate) (4.53 mmol) and 10 mg of 10% Pd/C in 3 mL of methanol was stirred at 25 °C for 1 h under  $\text{H}_2$  atmosphere. The mixture was filtered through a Celite bed and purified with preparative thin-layer chromatography ( $\text{CHCl}_3/\text{MeOH} = 20/1$ ) to give 37 mg of **26** as a white solid (100%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.06 (4H, d,  $J = 21.5$  Hz,  $2 \times -\text{CH}_2\text{P}-$ ), 3.66 (6H, s,  $2 \times -\text{CH}_3$ ), 3.70 (6H, s,  $2 \times -\text{CH}_3$ ), 6.55 (2H, s, Ph), 6.59 (1H, s, Ph);  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  27.09 (m), 33.48 (m); with proton decoupling off mode; MS (EI) ( $\text{M}^+$ ) 337; HRMS (EI) calcd 337.0844, found 337.0845.

**1-Aminobenzene-3,5-bis(methylphosphonic acid) Tetraammonium Salt (27).** To a solution of 35 mg of **26** (0.10 mmol) in 2 mL of anhydrous  $\text{CH}_3\text{CN}$  was added 0.14 mL of trimethylsilyl bromide (1.04 mmol) at 25 °C under  $\text{N}_2$  atmosphere. The mixture was stirred for 12 h, and the solvent was removed by  $\text{N}_2$  stream. The residue was partitioned between diethyl ether and 0.5 M ammonium bicarbonate solution. The aqueous fraction was applied to an ion-exchange chromatography column (Sephadex-DEAE A-25 resin) and eluted with a linear gradient (0.01 to 0.5 M) of 0.5 M ammonium bicarbonate. UV and HPLC were used to monitor the elution to give 35 mg of **27** (100%):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  2.84 (2H, d,  $J = 20.5$  Hz,  $-\text{CH}_2\text{P}-$ ), 2.93 (2H, d,  $J = 20.5$  Hz,  $-\text{CH}_2\text{P}-$ ), 6.60 (2H, s, Ar), 6.66 (H, s, Ar);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  18.85 (m), 20.29 (m); MS (FAB-) ( $\text{M} - \text{H}$ ) 280; HRMS (FAB-) calcd 280.0140, found

280.0127; HPLC retention time 4.1 min (purity >98%) using solvent system A, 7.8 min (>98% purity) using solvent system B.

**Tetraethyl 4-Aminophenylphosphonate (28).** A mixture of 0.69 g (4.0 mmol) of 4-bromoaniline, 0.57 mL (4.4 mmol) of diethyl phosphite, 0.61 mL (4.4 mmol) of triethylamine and 0.23 g (0.2 mmol) of tetrakis(triphenylphosphine)palladium(0) in 1 mL of anhydrous toluene was heated at 90 °C for 16 h under  $\text{N}_2$  atmosphere. After cooling, 10 mL of diethyl ether was added, and the precipitate was filtered off. The filtrate was concentrated and purified with preparative thin-layer chromatography ( $\text{CHCl}_3/\text{MeOH} = 20/1$ ) and crystallized in diethyl ether to give 0.18 g of **28** as a white solid (18%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.31 (6H, t,  $J = 6.8$  Hz,  $2 \times -\text{CH}_3$ ), 3.98–4.14 (4H, m,  $-\text{OCH}_2-$ ), 6.69, 6.71 (2H, 2d,  $J = 8.8$  Hz, Ar), 7.56, 7.61 (2H, 2d,  $J = 8.8$  Hz, Ar);  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  21.24 (m); MS (EI) ( $\text{M}^+$ ) 229; HRMS (EI) calcd 229.0868, found 229.0867.

**4-Aminophenylphosphonic Acid (29).** To a solution of 40 mg of **28** (0.18 mmol) in 2 mL of anhydrous  $\text{CH}_3\text{CN}$  was added 0.12 mL of trimethylsilyl bromide (0.88 mmol) at 25 °C under  $\text{N}_2$  atmosphere. The mixture was stirred for 12 h, and the solvent was removed by  $\text{N}_2$  stream. The residue was partitioned between diethyl ether and 0.5 M ammonium bicarbonate solution, and the aqueous fraction was purified with the same ion exchange column chromatography in general procedure to give 30 mg of **29** (99% yield):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  7.38, 7.39 (2H, 2d,  $J = 8.8$  Hz, Ar), 7.80, 7.84 (2H, 2d,  $J = 8.8$  Hz, Ar);  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  12.00 (m); MS (FAB+) ( $\text{M} + \text{H}$ ) 174; HRMS (FAB+) calcd 174.0320, found 174.0327; HPLC retention time 2.1 min (purity >98%) using solvent system A, 3.3 min (>98% purity) using solvent system B.

**Pharmacology.** Precautions must be taken to shield these P2X antagonists from light. In aqueous solution and upon exposure to light, azo-containing derivatives, such as **1** and **2**, had half-lives of several hours (G. Semple, unpublished observations). Both compounds were stable for days in solution when kept in the dark and for over a month as solids in clear bottles on the shelf.

**Antagonist Activity at P2X Receptors. At rat P2X<sub>1</sub>, P2X<sub>2</sub>, or P2X<sub>3</sub> receptors:** *Xenopus* oocytes were harvested and prepared as previously described.<sup>26</sup> Defolliculated oocytes were injected cytosolically with rat P2X<sub>1</sub>, P2X<sub>2</sub> or P2X<sub>3</sub> receptor cRNA (40 nL, 1  $\mu\text{g}/\text{mL}$ ), incubated for 24 h at 18 °C in Barth's solution and kept for up to 12 days at 4 °C until used in electrophysiological experiments.

ATP-activated membrane currents ( $V_h = -90$  mV) were recorded from cRNA-injected oocytes using the twin-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1–2 M $\Omega$  tip resistance) and current-recording microelectrodes (5 M $\Omega$  tip resistance) were filled with 3.0 M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 mL/min, at 18 °C) containing (mM) NaCl, 110; KCl, 2.5; HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[3-propanesulfonic acid]), 5;  $\text{BaCl}_2$ , 1.8, adjusted to pH 7.5.

ATP (at the  $\text{EC}_{70}$  values in  $\mu\text{M}$  for respective subtypes: P2X<sub>1</sub>, 1; P2X<sub>2</sub>, 10; P2X<sub>3</sub>, 3) was superfused over the oocytes for 60–120 s then washed out for a period of 20 min. For inhibition curves, data were normalized to the current evoked by ATP, at pH 7.5. Test substances were added for 20 min prior to ATP exposure; all compounds were tested for reversibility of their effects. The concentration required to inhibit the ATP response by 50% ( $\text{IC}_{50}$ ) was taken from Hill plots constructed using the formula:  $\log(I/I_{\text{max}} - I)$ , where  $I$  is the current evoked by ATP in the presence of an antagonist. Data are presented as mean  $\pm$  SEM ( $n = 4$ ) for data from different batches of oocytes.

**P2X<sub>7</sub> Receptor Channel Activation.** Cation channel activity of P2X<sub>7</sub> receptors was assayed as the ATP-induced loss of intracellular  $\text{K}^+$  in adherent HEK293 cells stably expressing recombinant human P2X<sub>7</sub> receptors. HEK293 cells were plated at  $5 \times 10^5$  cells/well on polylysine-coated wells of 12-well tissue culture chambers and grown to 90% confluence in DMEM (Dulbecco's modified eagle medium) plus 10% calf serum. The

growth medium was removed and replaced with 1 mL of divalent cation-containing saline consisting of 130 mM NaCl, 5 mM KCl, 20 mM NaHEPES (pH 7.5), 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 10 mM glucose, and 0.1% BSA. An alternative, divalent cation-free medium with the same basic composition but lacking the MgCl<sub>2</sub> and CaCl<sub>2</sub> was also tested. The cell monolayers were treated with the indicated concentrations of pyridoxal-phosphate derivative prior to the addition of 3 mM ATP (a maximal concentration of agonist under these ionic conditions). After a 10-min incubation at 37 °C, the assay medium was rapidly and completely aspirated and replaced with 1 mL 10% HNO<sub>3</sub>. After a 2-h extraction, the total K<sup>+</sup> content of the nitric acid extract was assayed by atomic absorbance spectrophotometry. The extent of ATP-induced K<sup>+</sup> efflux (plus or minus the various antagonists) was measured relative to the K<sup>+</sup> content of untreated cells. The K<sup>+</sup> content of control HEK cells bathed in divalent cation-containing saline was 531 ± 130 nmol K<sup>+</sup>/well (*n* = 3; with each experiment performed in duplicate for all tested conditions). Following a 10-min stimulation with ATP, the K<sup>+</sup> content was reduced to 141 ± 68 nmol/well. For cells bathed in divalent cation-free medium, the K<sup>+</sup> content was 512 ± 60 nmol K<sup>+</sup>/well, while the K<sup>+</sup> content of the ATP-stimulated cells was 135 ± 36 nmol/well. Relative efficacies of the different antagonists were calculated as the percentage inhibition of the ATP-induced K<sup>+</sup> loss in the absence of any pyridoxal-phosphate derivatives.

**P2X<sub>7</sub> Receptor-Dependent Pore Formation.** The pore-forming function of P2X<sub>7</sub> receptors was assayed as described previously in suspended HEK293 cells stably expressing recombinant human P2X<sub>7</sub> receptors.<sup>29</sup> Following trypsinization, the cells were suspended in the divalent cation-free saline described above. After transfer of a 1.5-mL aliquot of cell suspension to a thermostated (37 °C), stirred fluorimeter cuvette, 20 μM ethidium bromide was added. The cells were then treated with the indicated concentrations of pyridoxal-phosphate derivative prior to the addition of 300 μM ATP (a supramaximal concentration of agonist under these ionic conditions). The rates of ethidium influx were fluorimetrically monitored and calculated as described previously.<sup>29</sup> Relative efficacies of the different antagonists were calculated as the percentage inhibition of the ATP-induced rate of ethidium influx in the absence of any pyridoxal-phosphate derivatives.

**Phospholipase C Assay at P2Y Receptors.** P2Y<sub>1</sub> receptor-promoted stimulation of inositol phosphate formation by 2-MeSADP (10 nM) was measured in turkey erythrocyte membranes as previously described.<sup>27,28</sup> The values were averaged from 3–8 independent determinations. Briefly, 1 mL of washed turkey erythrocytes was incubated in inositol-free medium (DMEM; Gibco) with 0.5 mCi of 2-[<sup>3</sup>H]myo-inositol (20 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) for 18–24 h in a humidified atmosphere of 95% air 5% CO<sub>2</sub> at 37 °C. Erythrocyte ghosts were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EGTA) as described.<sup>26</sup> Phospholipase C activity was measured in 25 μL of [<sup>3</sup>H]inositol-labeled ghosts (ca. 175 μg of protein, 200–500000 cpm/assay) in a medium containing 424 μM CaCl<sub>2</sub>, 0.91 mM MgSO<sub>4</sub>, 2 mM EGTA, 115 mM KCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>, and 10 mM Hepes, pH 7.0. Assays (200 μL final volume) contained 1 μM GTPγS and the indicated concentrations of nucleotide analogues. Ghosts were incubated at 30 °C for 5 min, and total [<sup>3</sup>H]inositol phosphates were quantitated by anion-exchange chromatography as previously described.<sup>27,28</sup>

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