Complexities of Measuring Antagonist Potency at P2X<sub>7</sub> Receptor Orthologs

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ABSTRACT

The ability of P2 antagonists to affect agonist-stimulated fluorescent dye accumulation in cells expressing human, rat, or mouse P2X<sub>7</sub> receptors was examined. Several compounds, including pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), which was previously thought to be a weak P2X<sub>7</sub> receptor antagonist, possessed high potency (nanomolar IC<sub>50</sub>) at human and rat P2X<sub>7</sub> receptors. However, there were species differences in antagonist potency with PPADS, pyridoxal 5'-phosphate (P5P), and suramin. NN-(N,N-hexamethylene)amiloride (HMA) was also selective for human over rat P2X<sub>7</sub> receptors but potentiated responses at mouse P2X<sub>7</sub> receptors. Coomassie Brilliant Blue G (CBB) was a nonselective antagonist with high potency at mouse P2X<sub>7</sub> receptors (IC<sub>50</sub> ∼ 100 nM). All compounds were noncompetitive antagonists, and potency could only be quantified by measuring IC<sub>50</sub> values. These values were similar when determined against EC<sub>50</sub> concentrations of ATP or 2'- and 3'-O-(4-benzoylbenzoyl)-ATP and, for most compounds, only slightly (3- to 5-fold) affected by agonist concentration. However, IC<sub>50</sub> values for KN62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) and suramin, varied up to 25-fold depending upon agonist concentration.

Furthermore, IC<sub>50</sub> values for KN62 and OxATP were 10-fold lower at 22°C than at 37°C, whereas IC<sub>50</sub> values for PPADS, P5P, suramin, and OxATP were up to 20-fold lower in NaCl than in sucrose buffer. Potency estimates for CBB and PPADS decreased 5-fold in the presence of bovine serum albumin, possibly due to protein binding. Given the species differences, and the effects of assay conditions on antagonist potency, caution must be exercised when interpreting results obtained with the available antagonists.

The P2X<sub>7</sub> receptor is a nonselective cation channel gated by extracellular ATP (Surprenant et al., 1996) and is thought to equate with the previously described P2Z receptor (Gordon, 1986) responsible for the permeabilizing and cytolytic effects of ATP (Cockcroft and Gomperts, 1980; Heppel et al., 1985; Steinberg et al., 1987). This receptor exhibits a number of unusual properties, including the ability to change ionic selectivity during prolonged activation, such that it can exist in at least two distinct conductance states (Surprenant et al., 1996). Thus, with brief applications of ATP the P2X<sub>7</sub> receptor functions as a nonselective cation channel permeable to sodium and calcium ions (“small-pore” or “channel” state). However, after prolonged activation the ionic selectivity of the channel changes and the channel becomes permeable to molecules with molecular weights of up to 800 (“large-pore” state).

A number of studies have described the effects of antagonists on endogenous P2Z receptors in various cell lines. Thus, Coomassie Brilliant Blue G (CBB), cibacron blue, and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid have been reported as antagonists of P2Z receptors in rat parotid acinar cells (Soltoff et al., 1989, 1993), whereas suramin, 5-(N,N-hexamethylene)amiloride (HMA), and cibacron blue have been shown to block P2Z receptors in human lymphocytes (Wiley et al., 1993). There is only limited information on the effects of P2 antagonists such as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and suramin at recombinant P2X<sub>7</sub> receptors (Surprenant et al., 1996; Rassendren et al., 1997; Chessell et al., 1995a,b), although recently the effects of 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62; Humphreys et al., 1998) and CBB (Jiang et al., 2000) have been studied in detail. However, in most of these studies antagonist IC<sub>50</sub> values have been estimated without determining if compounds were competitive or noncompetitive antagonists or if agonist concentration affected IC<sub>50</sub> estimates. Furthermore, there are considerable variations in antagonist equilibrium times in most studies, and in some studies ionic substitutions to replace NaCl have been used (Wiley et al., 1993; Michel et al., 1999) or assays have been performed with reduced concentrations of calcium or magnesium ions (Soltoff et al., 1993;
Wiley et al., 1993; Humphreys et al., 1998; Michel et al., 2000).

The failure to study antagonist effects in detail is partly due to the low potency of agonists at the P2X7 receptor, which makes construction of complete agonist concentration-effect curves (CECs) difficult. However, we have previously found that replacement of extracellular NaCl with sucrose results in a marked increase in agonist potency and that this enables complete agonist CECs to be obtained (Michel et al., 1999).

Furthermore, using this approach it was possible to examine antagonist effects on the human P2X7 receptor by measuring agonist-stimulated influx of the DNA-binding dye YO-PRO-1 (Michel et al., 2000).

A major limitation of this methodology is that colored or fluorescent compounds can interfere with measurements, thereby limiting the range of compounds that can be examined. In the present study we have extended this methodology by studying adherent cells. This provides an increase in assay sensitivity and enables colored/fluorescent compounds to be examined. Furthermore, since there is evidence for species differences in the effects of KN62 at the P2X7 receptor (Humphreys et al., 1998), we have examined antagonist effects at the rat, human, and mouse P2X7 receptors to determine whether these species differences are observed with other antagonists. Finally, since several aspects of the methodology rely upon the use of ionic substitutions, we have determined the extent to which assay conditions affect estimates of antagonist potency.

Experimental Procedures

Tissue Culture. All studies were performed using HEK293 cells transfected by electroporation of cDNAs encoding rat (Surprenant et al., 1996), human (Rassendren et al., 1997), or mouse (Chessell et al., 1998a) P2X7 receptors. The cell lines were selected for stable expression of the recombinant receptor and were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 containing 10% fetal bovine serum and supplemented with 0.6 mg · ml⁻¹ genetin sulfate. Cells were grown as monolayer cultures at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ and were harvested when confluent by incubation in trypsin/EDTA solution and diluted 10-fold into fresh growth media.

Assay Buffers. The assay buffers used to measure ethidium or YO-PRO-1 accumulation comprised (in mM): HEPES 10, N-methyl-D-glucamine 5, KCl 5.6, t-glucose 10, CaCl₂ 0.5 (pH 7.4) and were supplemented with either 280 mM sucrose (sucrose buffer) or 140 mM NaCl (NaCl buffer). In some studies 1 mM MgCl₂ and 1 mM CaCl₂ were included in the NaCl buffer.

YO-PRO-1 Accumulation. The methods for studying YO-PRO-1 accumulation have been described previously (Michel et al., 1999). Briefly, cells were harvested in trypsin/EDTA solution, collected by centrifugation (200g for 5 min), and washed once in ice-cold assay buffer before resuspending in assay buffer at 37°C. Cell suspensions were added to 96-well Costar 1/2-well plates (approximately 40,000 cells well⁻¹; Costar, High Wycombe, Bucks, UK) containing the antagonist under study. After 30 min at 37°C 2' and 3'-O-(4-benzoylbenzoyl)-ATP (BzATP) and YO-PRO-1 (1 μM) were added, and changes in YO-PRO-1 fluorescence were monitored at 37°C in a Canberra Packard (Pangbourne, Berks, UK) Fluorocount 96-well plate fluorimeter (excitation wavelength of 485 nm and emission wavelength of 530 nm). In these experiments agonist incubation times of 5, 10, or 40 min were utilized when studying rat, human, or mouse P2X7 receptors, respectively. At these times approximately 10 to 50% of maximal agonist-stimulated YO-PRO-1 accumulation had occurred. In studies to determine the time course of agonist-stimulated YO-PRO-1 accumulation, responses were recorded, in the absence of antagonist, at various times after agonist and YO-PRO-1 addition.

Ethidium Accumulation in Adherent Cells. Cells expressing rat, mouse, or human recombinant P2X7 receptors were harvested and added to poly(l-lysine)-pretreated 96-well plates (Costar, UK) and grown until they formed a confluent monolayer (18–24 h).

Cells were washed twice with 350 μl of assay buffer before addition of 100 μl of assay buffer containing the antagonist. After 30-min incubation, agonist and ethidium (100 μM final assay concentration) were added and incubations continued until approximately 20 to 80% of maximal dye accumulation occurred (see below). The ethidium solution was removed, and cellular accumulation of ethidium was determined by measuring fluorescence from below the plate with a Canberra Packard Fluorocount (excitation wavelength of 530 nm and emission wavelength of 620 nm). In studies to determine the time course of agonist-stimulated ethidium accumulation, responses were recorded, in the absence of antagonist, at various times after agonist and ethidium addition.

The rates of agonist-stimulated ethidium accumulation vary considerably between the three species and are also affected by assay buffer and temperature (see Results). Consequently, agonist exposure times were adjusted to ensure that measurements of agonist and antagonist potency were made when approximately 20 to 80% of the maximal agonist-stimulated dye accumulation had occurred. Thus, in sucrose buffer at 37°C, incubation times with agonist were 1, 2, and 5 min when studying rat, human, and mouse receptors, respectively. In sucrose buffer at 22°C, incubation times with agonist were 1, 2, and 10 min when studying rat, human, and mouse receptors, respectively. In NaCl buffer at 22°C incubation, times with agonist were 2 and 5 min when studying rat and human receptors, respectively. It should be noted that estimates of agonist and antagonist potency do not vary significantly when determined after various time of agonist exposure [Michel et al. (2000) and A. D. Michel, unpublished observations].

Data Analysis. In studies with the antagonists, the agonist CECs are presented in the absence and presence of the antagonist. For graphical purposes the data are presented as a percentage of the maximal response obtained in the control group without removing basal fluorescence values. In all cases, mean data are presented and lines drawn through the data do not represent lines of best fit. In the time course studies, basal fluorescence was subtracted and the data are presented as a percentage of the maximal specific BzATP-stimulated fluorescence.

In the majority of studies, complete agonist CECs were determined in the absence or presence of various concentrations of antagonist. Individual CECs from each experiment were then fitted to a four-parameter logistic function using either Prism 3 (GraphPad, San Diego, CA) or SigmaPlot (SPSS Science, Chicago, IL) to determine the maximum and minimum responses and to calculate the pEC₅₀ value and the Hill slope. The pEC₅₀ for each agonist was also determined at each concentration of agonist. These inhibition curves were fitted to a two-parameter logistic function using GraphPad Prism to calculate the pIC₅₀ value and the Hill slope. For this analysis, the maximal response was fixed at that obtained in the absence of antagonist, at each concentration of agonist, whereas the minimum response was fixed at the basal accumulation measured in the absence of agonist and antagonist. In the tables, pIC₅₀ values are presented against a concentration of agonist that was close to the EC₅₀ value determined in the control cells. Due to the high Hill slopes for the agonist CECs (2.1–6.7), this concentration of agonist was not always the same; therefore, within any study the concentrations of agonist employed ranged from an EC₂₀ to an EC₅₀ concentration. However, this represented, at most, a 2-fold variation in agonist concentration.

All statistical comparisons, determinations of pIC₅₀ values, and calculations of agonist EC₅₀ values were performed on the raw,
nontransformed data. The data are the mean ± S.E.M. of 3 to 11 experiments.

To illustrate the potential dependence of antagonist potency upon agonist concentration, theoretical CECs were simulated in the presence of various concentrations of an assumed competitive antagonist. Thereafter, pIC$_{50}$ values for the antagonist were calculated at various agonist concentrations using GraphPad Prism 3. For these simulations, agonist responses were modeled assuming the following relationship between fractional response ($R$), agonist concentration ($A$), antagonist concentration ($B$), agonist potency (EC$_{50}$), and antagonist affinity (KB):

$$R = \frac{A}{A + \text{EC}_{50}(1 + \frac{B}{\text{KB}})}$$

For simulations involving the human P2X$_7$ receptor, an agonist EC$_{50}$ of 0.25 μM and antagonist KB of 10 nM were assumed, whereas, for simulations involving the mouse P2X$_7$ receptor, an agonist EC$_{50}$ of 32 μM and antagonist KB of 1 μM were assumed (see Fig. 3A).

To compare differences in pIC$_{50}$ values obtained with each antagonist under the different assay conditions, a one-way ANOVA with Tukey’s post analysis test was used (GraphPad Prism 3). The data in Tables 1, 2, and 3 were utilized in this analysis.

Materials.

All tissue culture reagents were from Gibco (Paisley, Scotland). ATP was obtained from Promega (Southampton, UK). KN62 was from Seikagaku (Rockville, MD). YO-PRO-1 was obtained from Molecular Probes (Eugene, OR). Alphazurine, Brilliant black, BzATP, bovine serum albumin (BSA), Chicago sky blue, CBB, ethidium bromide, HMA, periodate oxidized ATP (OxATP), PPADS, pyridoxal 5’ phosphate (P5P), Reactive black 5, Reactive blue 4, Remazol brilliant blue, and suramin were obtained from Sigma/RBI (Poole, UK). All other chemicals were of Anal-R grade and were obtained from Fisons (Loughborough, UK). KN62 and P5P were prepared as described previously (Michel et al., 2000). HMA was dissolved at 30 mM in dimethyl sulfoxide before dilution into assay buffer. Dimethyl sulfoxide had no effect on the BzATP CECs at concentrations of up to 1% (A. D. Michel, unpublished data). Other drugs were dissolved in assay buffer.

Results

Kinetics of Agonist-Stimulated DNA-Binding Dye Accumulation

Initial studies were undertaken to examine BzATP-stimulated dye accumulation in either suspensions or monolayers of cells expressing the P2X$_7$ receptors to identify conditions for studying the effects of antagonists under similar conditions.

Cell Suspensions. In cell suspensions, the only condition under which the three species orthologs could be examined was in sucrose buffer at 37°C. In this buffer, BzATP-stimulated YO-PRO-1 accumulation was most rapid in cells expressing the rat P2X$_7$ receptor and slowest in cells expressing the mouse P2X$_7$ receptor (Fig. 1A). When assessed at a time when 50% of maximal accumulation had occurred (t$_{1/2}$), BzATP stimulated approximately 5-, 4-, and 2.5-fold increases over basal accumulation in studies on the rat, human, and mouse P2X$_7$ receptors, respectively. At 22°C, there was no detectable BzATP-stimulated YO-PRO-1 accumulation in suspensions of cells expressing the mouse P2X$_7$ receptor over a 3-h period, whereas in cells expressing the rat and human P2X$_7$ receptors, BzATP-stimulated YO-PRO-1 accumulation was very slow with an approximate doubling over basal levels after 30 min and 2 h, respectively (data not shown). There was no detectable BzATP-stimulated ethidium accumulation in cell suspensions, probably due to the very high fluorescence of the ethidium solution (data not shown).

In NaCl-containing buffer, it was only possible to measure BzATP-stimulated YO-PRO-1 accumulation at 37°C.
and in cells expressing rat or human P2X7 receptors (data not shown).

Adherent Cells. In adherent cells, BzATP-stimulated YO-PRO-1 (Fig. 1D) or ethidium accumulation (Fig. 1B) could be measured at all three species orthologs in sucrose buffer at 37°C. For all three species orthologs the BzATP-stimulated dye accumulation, measured at time equivalent to the $t_{1/2}$, was approximately 10- to 30-fold greater than the basal values. The greater signal-to-noise ratio in these studies using adherent cells was presumably due to removal of the dye from above the cells. Furthermore, since measurements were made from below the plate, it is likely that fluorescence in the cell monolayer was measured rather than fluorescence from residual amounts of dye left in the solution above the cells.

BzATP-stimulated ethidium accumulation in adherent cells at 37°C was more rapid than BzATP-stimulated YO-PRO-1 accumulation determined in suspensions of cells (Fig. 1, A and B). However, this probably reflects the use of a higher concentration of ethidium (100 μM) than YO-PRO-1 (1 μM), since the rates of BzATP-stimulated YPRO-1 accumulation were similar when studied in adherent cells and cell suspensions (Fig. 1D). In contrast to studies on YO-PRO-1 accumulation in suspensions of cells, BzATP-stimulated ethidium accumulation could be measured at the three species orthologs when studied at 22°C in sucrose buffer (Fig. 1C).

In NaCl buffer, it was possible to measure BzATP-stimulated YO-PRO-1 accumulation in adherent cells expressing rat or human P2X7 receptors at either 22 or 37°C, although there was no detectable dye accumulation in cells expressing the mouse P2X7 receptor when using BzATP concentrations of up to 256 μM.

Although the maximal rates of BzATP-stimulated YO-PRO-1 and ethidium accumulation in sucrose buffer were very different, the potency estimates for BzATP determined using the two dyes were similar. Thus, in studies on the rat, human, and mouse P2X7 receptors, pEC50 values for BzATP to stimulate YO-PRO-1 accumulation in cell suspensions were $6.8 \pm 0.14$, $6.3 \pm 0.13$, and $4.7 \pm 0.07$, respectively. The values at each species ortholog were not significantly different ($P > 0.05$, Student’s t test) to the respective values of $7.1 \pm 0.09$, $6.3 \pm 0.06$, and $4.9 \pm 0.09$ to stimulate ethidium accumulation in adherent cells expressing the rat, human, and mouse P2X7 receptors, respectively. These pEC50 values were estimated at time points equivalent to the $t_{1/2}$ for each species ortholog. We have previously shown that estimates of agonist potency at the human receptor are similar when determined at various times after addition of BzATP (Michel et al., 2000). In the present study, for each method used, agonist potency estimates were similar when utilizing time points up to three times the $t_{1/2}$ value (data not shown). Consequently, in all subsequent studies, agonist potency was determined at a time when approximately 20 to 80% of maximal dye accumulation occurred (see Experimental Procedures).

Species Differences in Antagonist Effects on BzATP-Stimulated YO-PRO-1 Accumulation in Cell Suspensions

It was only possible to perform comparative studies on the effects of antagonists on BzATP-stimulated YO-PRO-1 accumulation in cell suspensions at 37°C and in sucrose buffer. The effects of antagonists on BzATP-stimulated YO-PRO-1 accumulation in cells expressing the human P2X7 receptor under these conditions have been described (Michel et al., 2000). Similar studies were performed on the rat and mouse P2X7 receptors. Only PPADS, P5P, OxATP, and KN62 could be examined, since CBB, suramin, and many of the colored dyes could affect the fluorescence of YO-PRO-1/nucleotide mixtures.

The effects of PPADS (Fig. 2A), OxATP, and P5P at the rat
P2X7 receptor were qualitatively similar to those observed at the human P2X7 receptor (Michel et al., 2000). Thus, these compounds produced a concentration-dependent reduction in the maximal response with a slight (2- to 10-fold) reduction in agonist EC50. The threshold concentrations of PPADS (Fig. 2A), P5P, and OxATP for blocking the rat P2X7 receptor were approximately 100 nM, 0.3 to 1 μM, and 10 μM, respectively, and were approximately 3- to 10-fold higher than the threshold concentrations of 10 nM, 100 nM, and 1 to 3 μM, respectively, determined at the human P2X7 receptor (Michel et al., 2000). In contrast, KN62 was ineffective at the rat P2X7 receptor at concentrations of up to 10 μM (Fig. 2B), whereas at the human P2X7 receptor the threshold concentration was 10 to 30 nM (Michel et al., 2000). At the mouse P2X7 receptor, PPADS, P5P, and OxATP were much weaker antagonists than at the human P2X7 receptor (approximately 30- to 1000-fold) with threshold concentrations of approximately 3 to 10 μM, 3 to 10 μM, and 30 to 100 μM, respectively (Fig. 2C). KN62 was an antagonist of the mouse P2X7 receptor with a threshold concentration of 0.3 to 1 μM, although it also slightly reduced the basal responses (Fig. 2D).

Due to the complex nature of the antagonist effects, these data could not be analyzed using Schild analysis. Therefore, pIC50 values were determined. A major limitation of this approach is that pIC50 values could depend on agonist concentration. Thus, for a competitive antagonist, the pIC50 should decrease as the agonist concentration is increased (Fig. 3A), although the relationship between pIC50 and agonist concentration is not a simple linear one. In the case of a noncompetitive antagonist of an ion channel, for which there should be no receptor reserve, pIC50 values should not change with agonist concentration.

To determine experimentally the extent to which agonist concentration affected antagonist potency, pIC50 values for each antagonist were determined at each agonist concentration. For PPADS (Fig. 3A), P5P, and OxATP, pIC50 values did not vary markedly (3- to 10-fold) with agonist concentration at the three species orthologs. In contrast, for KN62 the pIC50 values for the human P2X7 receptor varied from 7.6 when using an EC50 concentration of BzATP to 6.2 when using a maximum concentration of BzATP (Fig. 3A).

To provide a species comparison of antagonist potency, the pIC50 values of the antagonists for the three species orthologs were determined against an approximate EC50 concentration of agonist, since, at this concentration, the dependence of antagonist pIC50 on agonist concentration should be minimized (Fig. 3A). On the basis of these pIC50 values, PPADS, P5P, and OxATP possessed 20- to 500-fold higher potency for human than for mouse P2X7 receptors and possessed intermediate potency at the rat P2X7 receptor (Table 1). The selectivity of PPADS, P5P, and OxATP for human over mouse P2X7 receptors was maintained over a range of agonist concentrations (data not shown). Furthermore, the conclusions on antagonist selectivity are consistent with esti-
Antagonist Effects Determined in Adherent Cells Using Ethidium Influx

A major limitation of the studies using cells in suspension is the problem of background fluorescence and quenching of the fluorescence signal by colored compounds. In addition, for the mouse receptor the kinetics of the response were slow and the signal-to-noise ratio was poor and quite variable. All of these issues could be resolved by performing studies on adherent cells (see above), and this enabled additional antagonists such as suramin and CBB to be examined.

The effects of the antagonists on BzATP-stimulated ethidium accumulation, measured in sucrose buffer at 37°C, were similar to those obtained using YO-PRO-1. Thus, at all three species PPADS, P5P, and OxATP behaved as noncompetitive antagonists reducing the maximal response and decreasing agonist potency slightly (data not shown). CBB was a relatively potent noncompetitive antagonist of all three species (Fig. 4). Suramin was also an antagonist at all three species producing a decrease in agonist potency and a reduction in the maximal response (Fig. 4D, data for mouse P2X7 receptor).

To compare potency estimates between the species, pIC50 values were again determined using an approximate EC50 concentration of BzATP at each species ortholog (Table 2). The pIC50 values for PPADS, P5P, and OxATP were similar to those determined in studies measuring BzATP-stimulated YO-PRO-1 accumulation (Table 1) and confirmed the selectivity of these agents, particularly for the human as opposed to mouse P2X7 receptors. Similarly, KN62 was highly selective for the human as compared with rat P2X7 receptors. As with studies on YO-PRO-1 accumulation, pIC50 values for PPADS, P5P, and OxATP at the human P2X7 receptor did not vary markedly (less than 2-fold) with agonist concentration, whereas pIC50 values for KN62 at the human and mouse P2X7 receptors decreased (5- to 6-fold) with increasing concentration of agonist (Fig. 3, B and D). CBB possessed similar potency at the three species homologs and was the most potent antagonist of the mouse P2X7 receptor (Table 2). Suramin displayed similar potency at the three species homologs, although pIC50 values showed considerable variation (10- to 40-fold) with agonist concentration (Fig. 3, B–D).

Fig. 4. Antagonist effects on BzATP-stimulated ethidium accumulation in adherent HEK293 cells expressing recombinant P2X receptors. Adherent cells were incubated in sucrose buffer at 37°C with CBB (A–C) or suramin (D) for 30 min before addition of BzATP and ethidium (100 μM). Ethidium fluorescence was measured 2 min (A, human P2X7 receptor), 2 min (B, rat P2X7 receptor), or 10 min (C and D, mouse P2X7 receptor) after agonist addition. The data have been normalized to the maximal response measured in the control cells for each experiment (% of control maximum). Basal accumulation has not been removed and is represented by the leftmost data point in each figure. The data are the mean ± S.E.M. of three to six determinations. Lines drawn through the data points do not represent lines of best fit.

TABLE 1

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
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<td>5.2 ± 0.1**</td>
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<td>&lt;5*</td>
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<tr>
<td>OxATP</td>
<td>5.5 ± 0.1</td>
<td>4.6 ± 0.1*</td>
<td>4.2 ± 0.1*</td>
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</table>

* Value significantly different to that determined at the human P2X7 receptor or, |
| ** significantly different to that determined at rat P2X7 receptor (P < 0.05; one-way ANOVA and Tukey's post test). |
Antagonist pIC\textsubscript{50} values to inhibit BzATP-stimulated ethidium accumulation

Studies were conducted using adherent cells expressing human, rat, or mouse P2\textsubscript{X}\textsubscript{7} receptors in either sucrose (Suc) or NaCl buffer at 22 or 37°C. Data are pIC\textsubscript{50} values (mean ± S.E.M. of 3–11 experiments) determined against an EC\textsubscript{50} concentration of BzATP.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Buffer</th>
<th>Temperature</th>
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<th>Rat</th>
<th>Mouse</th>
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</tr>
<tr>
<td></td>
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<tr>
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<tr>
<td></td>
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<td>22</td>
<td>6.8 ± 0.2</td>
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</tr>
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<td></td>
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<td>5.8 ± 0.1*</td>
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<tr>
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<td>&lt;5</td>
<td>6.4 ± 0.1</td>
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<tr>
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<tr>
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<td>4.8 ± 0.1*</td>
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<tr>
<td>CBB</td>
<td>Suc</td>
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<td>7.0 ± 0.3</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Suc</td>
<td>22</td>
<td>6.2 ± 0.2*</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>22</td>
<td>5.8 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.
\* Significant difference between values in sucrose at 37 and 22°C or, \# between values in sucrose and NaCl at 22°C (P < 0.05; one-way ANOVA and Tukey’s post test).

Effects of Assay Buffer and Temperature on Antagonist Potency

The studies presented above were performed in sucrose buffer, since it was possible to construct complete CECs at all three species orthologs in this buffer. However, as sucrose is not a physiological buffer, additional studies were undertaken to assess antagonist effects in NaCl buffer. In addition, many studies on P2X receptors are performed at room temperature (22°C), whereas in the present, and many other studies, 37°C has been utilized. Consequently, the effect of temperature on antagonist potency was also examined.

The antagonist effects of the compounds at room temperature or in NaCl buffer were qualitatively similar to their effects in sucrose buffer. Thus, none of the compounds were simple competitive antagonists, since they decreased maximal responses and produced either no, or only a small, decrease in BzATP EC\textsubscript{50} (data not shown).

Effect of Temperature. There was little effect of temperature (<3- to 4-fold differences) on pIC\textsubscript{50} values for PPADS, P5P, or suramin at the human P2\textsubscript{X}\textsubscript{7} receptor when studied in either sucrose buffer (Table 2) or NaCl buffer (data not shown). However, pIC\textsubscript{50} values for KN62 at the human P2\textsubscript{X}\textsubscript{7} receptor were approximately 10-fold lower when studied at 22°C than at 37°C (Table 2). Potency estimates for OXATP at the rat and mouse P2\textsubscript{X}\textsubscript{7} receptor were also 3- to 20-fold lower in sodium than in sucrose containing buffer. The effect of sodium on antagonist potency at the human P2\textsubscript{X}\textsubscript{7} was similar at both 22 and 37°C (data not shown).

Effect of Divalent Cations of Antagonist Potency. The potency estimates for PPADS and suramin determined at the rat P2\textsubscript{X}\textsubscript{7} receptor were much greater than the respective IC\textsubscript{50} values of 45 to 60 \mu M and >300 \mu M, determined in electrophysiological studies on this receptor (Surprenant et al., 1996). Since the later studies were conducted at 22°C in NaCl buffer in the presence of calcium and magnesium ions, the effects of PPADS and suramin were examined at the rat P2\textsubscript{X}\textsubscript{7} receptor at 22°C in NaCl buffer containing 1 mM Mg\textsubscript{2+} and 1 mM Ca\textsubscript{2+}. In this buffer the potency of both PPADS and suramin was still greater than determined in electrophysiological studies (Fig. 5). The pIC\textsubscript{50} values for PPADS and suramin, determined against an EC\textsubscript{50} concentration of BzATP, were 5.8 ± 0.1 and 4.7 ± 0.1, respectively, and were not significantly different (P > 0.05; one-way ANOVA and Tukey’s test) to those determined in NaCl buffer with 0.5 mM Ca\textsubscript{2+} (Table 2).

Antagonist Effects on Responses to ATP

It has been reported that potency estimates for KN62 differ when determined against maximal concentrations of ATP and BzATP (Gargett et al., 1997). Consequently, antagonist effects on ATP- and BzATP-stimulated ethidium accumulation were determined at the human and rat P2\textsubscript{X}\textsubscript{7} receptors to determine whether antagonist potency is dependent on the agonist used. These studies were performed in NaCl buffer at 22°C. The pIC\textsubscript{50} values determined against an EC\textsubscript{50} concentration of ATP at the rat and human P2\textsubscript{X}\textsubscript{7} receptors were not significantly different (P > 0.05; one-way ANOVA and Tukey’s test) to the respective values determined at the rat and human P2\textsubscript{X}\textsubscript{7} receptors when using an EC\textsubscript{50} concentration of BzATP (cf. Tables 2 and 3). Similar results were
Effects of Polysulfonated Dyes

Given the high potency of CBB, a range of other polysulfonated dyes were examined at the three species homologs of the P2X7 receptor in sucrose buffer at 22°C. The polysulfonated dyes alazhumazine, Brilliant black, Chicago sky blue, cibacron brilliant red, Reactive blue 4, Reactive black 5, and remazol brilliant blue all exhibited similar effects to CBB in that they produced marked reductions in maximal responses with little or no effect on agonist EC50 (data not shown). When comparing pIC50 values against an EC50 concentration of BzATP (values in parentheses represent pIC50 values at human, rat, and mouse P2X7 receptors, respectively), cibacron brilliant red (5.2, 5.5, 6.0) and Chicago sky blue (5.8, 5.9, 6.6) were approximately 6-fold selective for mouse over human P2X7 receptors, whereas remazol brilliant blue (6.3, 5.5, 5.9) was slightly (3- to 6-fold) more potent at the human than at the rat or mouse P2X7 receptors. It is unlikely that the effects of the dyes reflect quenching of fluorescence signals as the two black dyes, Reactive black 5, and Brilliant black, which should exhibit the greatest quenching effect, possessed the lowest pIC50 values of the various dyes examined (pIC50 values 4.8–5.7).

Effects of HMA

In sucrose buffer at 22°C, HMA was an antagonist of the human P2X7 receptor producing a reduction in agonist EC50 and a slight reduction in the maximal response (Fig. 6). Effects at the rat P2X7 receptor were less pronounced, whereas at the mouse P2X7 receptor HMA produced complex effects and was an antagonist at higher concentrations but at low concentrations produced an increase in responses (Fig. 6). This latter effect was only observed at 8 and 16 μM BzATP. This behavior was not observed for any of the other antagonists studied. When comparing pIC50 values in sucrose buffer, HMA appeared slightly selective for human (pIC50 = 5.3 ± 0.1) over rat receptors (pIC50 = 4.9 ± 0.1), although the selectivity was not marked, especially since the pIC50 value at the human P2X7 receptor decreased with agonist concentration (data not shown). In NaCl buffer, the potency of HMA at the human P2X7 receptor decreased (pIC50 = 4.5 ± 0.1), whereas at the rat receptor the compound had no effect at concentrations up to 100 μM.

Effect of BSA on Antagonist Potency

Many tissue culture studies are performed in the presence of serum and so the extent to which antagonists may be protein bound was assessed by examining the effect of BSA on antagonist potency. These studies were conducted using cells expressing the human P2X7 receptor and were performed in NaCl buffer at 22°C. Inhibition curves were constructed in the presence and absence of 0.1% BSA. Potency estimates for P5P, suramin and KN62 were not affected by 0.1% BSA whereas those for CBB and PPADS were reduced 5.8 ± 0.4 and 5.3 ± 0.6 fold (n = 3) in the presence of BSA.

Discussion

The findings of this study were 2-fold. First, that many P2-receptor antagonists are also potent P2X7 receptor antagonists and exhibit considerable species selectivity, with this
latter observation even extending to OxATP. Second, that none of these compounds were competitive antagonists and potency estimates were markedly affected by assay conditions creating a significant source of variability in data between various laboratories.

Quantitative Studies on P2X7 Receptors. The use of fluorescent dyes to examine P2X7 receptor function has been widely used [see Michel et al. (2000) for references]. This methodology has now been adapted for use with adherent cells, and this has produced a marked improvement in assay sensitivity and enabled a greater range of antagonists to be examined.

Our main goal was to determine the extent of species differences in antagonist potency. A number of structurally diverse compounds were examined, although none were competitive antagonists because they reduced maximal responses. Although this was expected with the irreversible antagonist OxATP (Murgia et al., 1993), it was not expected with PPADS, P5P, or suramin, since they are reversible antagonists and probably interact with the ATP-binding site (Michel et al., 2000). However, these compounds are slowly reversible (Michel et al., 2000). Consequently, their dissociation may be minimal during measurement of agonist responses, resulting in their acting as pseudoirreversible antagonists (Kenakin, 1993). This may be overcome by extending agonist equilibration times, but the physiological relevance of this would be uncertain, as it is unlikely that the high ATP concentrations required to activate P2X7 receptors would persist for such prolonged periods in vivo.

The noncompetitive behavior of the antagonists prevented the use of Schild analysis to determine pA2 values and necessitated calculation of pIC50 values. A limitation of this approach is that pIC50 values could be agonist concentration-dependent. However, with the exception of KN62 and suramin, pIC50 values did not vary markedly with agonist concentration. Furthermore, by determining pIC50 values against EC50 concentrations of agonist, potential effects of agonist concentration on antagonist potency were minimized.

PPADS, P5P, and OxATP were 20- to 500-fold selective for human over mouse receptors, whereas KN62 exhibited 500-fold selectivity for human over rat receptors. Several other compounds exhibited 3- to 10-fold selectivity between species but, considering the limitations of using pIC50 values, the significance of this selectivity could be questioned. However, the selectivity of PPADS, P5P, OxATP, and KN62 indicates major species differences in antagonist potency. Since the amino acid sequence of the species homologs is known, it may be possible to use this information to identify the binding sites for the antagonists.

Studies on P2X7 receptors are often conducted under widely varying conditions, so we examined the effect of assay conditions on antagonist potency. Potency estimates for PPADS, P5P, and suramin were temperature-insensitive, whereas those for OxATP, KN62, and CBB at the human receptor were reduced at 22°C. The reason for this differential temperature sensitivity is not known but has implications when using these compounds to discriminate between receptor types. Furthermore, although NaCl did not affect potency estimates for KN62 or CBB, those for PPADS, P5P, OxATP, and suramin were 3- to 10-fold lower in NaCl than in sucrose buffer. This was not unexpected, since these compounds probably interact with the ATP-binding site (Michel et al., 1999) and so could be subject to the same ionic modulation by NaCl as agonists (Michel et al., 2000).

Antagonist potency estimates were not agonist-dependent when estimated against EC50 concentrations of ATP or BzATP. Although this may appear to contrast with studies by Gargett et al. (1997), in which IC50 values for KN62 were different when using ATP and BzATP, it should be noted that they utilized maximal concentrations of each agonist. Furthermore, pIC50 values for KN62 exhibited considerable concentration dependence in this study. These observations stress the need for caution when using KN62 to define P2X7 receptors. Finally, pIC50 values for PPADS and CBB were reduced in the presence of 0.1% BSA. This is probably due to
protein binding and could exert an important influence on potency estimates in vivo or in culture systems containing serum.

In addition to providing general information on antagonist interactions with P2X7 receptors, this study has also provided specific information on a number of P2 receptor antagonists that deserves comment.

**OxATP.** OxATP is the prototypic P2X7 receptor antagonist, but this study demonstrates that it is selective for the human P2X7 receptor. This may explain the variations in OxATP concentrations used in previous studies. Thus, concentrations of OxATP required to block mouse and human P2X7 receptors were 300 μM (Murgia et al., 1993) and 10 μM (Wiley et al., 1994), respectively. In addition to species differences, NaCl and temperature also affected the potency of OxATP. The reasons for these effects were not studied further, however, considering that sensitivity to OxATP is often used to define P2X7 receptors, these issues illustrate the need for caution when using this compound.

**PPADS and P5P.** PPADS and P5P were potent antagonists of human and rat P2X7 receptors but weak antagonists of the mouse P2X7 receptor. The reason for the lower potency of PPADS in previous studies (Surprenant et al., 1996; Rassendren et al., 1997) is not known but may reflect the need for prolonged equilibration times with PPADS (Chessell et al., 1998b; Michel et al., 2000). Irrespective of the reason, it seems inappropriate to consider PPADS as a weak antagonist of the P2X7 receptor.

**KN62.** This study confirmed the selectivity of KN62 for human P2X7 receptors (Chessell et al., 1998a; Humphreys et al., 1998) and demonstrated that KN62 can also block mouse P2X7 receptors. The potency of KN62 at the human P2X7 receptor was greatly reduced at 22°C, which has implications when using this compound to define P2X7 receptors in dye accumulation studies. Interestingly, at room temperature, 10 nM KN62 blocks human P2X7 receptor-mediated currents (Humphreys et al., 1998), but 300 nM KN62 was required to block ethidium accumulation. The reasons for this discrepancy are not known, but we have noted that several other compounds also exhibit similar selectivity and differentiate between the presumed "channel" and "large-pore" forms of the receptor studied in electrophysiology and dye accumulation studies, respectively (Michel et al., 2000).

**CBB.** CBB is a P2X7 receptor antagonist (Soltoff et al., 1989) that is highly selective for P2X7 over other P2X receptor types (Jiang et al., 2000). In this study CBB was an antagonist at rat, human, and mouse P2X7 receptors. The high potency at mouse P2X7 receptors is of interest given the low potency of other antagonists at this ortholog. The concentration of CBB that blocked BzATP-stimulated dye accumulation at rat P2X7 receptors (100–300 nM) was higher than required to block ATP-evoked currents (10 nM; Jiang et al., 2000). As with KN62, this may reflect differential effects on the "channel" and "large-pore" forms of the receptor. However, since CBB can be protein-bound, the lower potency in this study could reflect compound depletion by the cell monolayer, an effect that should be less pronounced in electrophysiological studies where compounds are superfused and cell density is lower. Finally, the selectivity of CBB for rat over human receptors at 22°C (Jiang et al., 2000) was confirmed, although our results show that this selectivity is not observed at 37°C.

**HMA.** HMA blocks human but not rat P2X7 receptors (Wiley et al., 1994; Surprenant et al., 1996) and potentiates responses at mouse P2X7 receptors (Chessell et al., 1997). Similar results were obtained in this study, although potentiation of responses at the mouse receptor was only observed at low concentrations of BzATP. HMA is unlikely to be of use in characterizing P2X7 receptors due to its low potency, but its ability to potentiate responses at the mouse receptor is of interest, especially as this property has also been reported for tenidap, a compound with potent anti-inflammatory effects (Sanz et al., 1998).

**Conclusion.** A wide range of P2 antagonists are potent noncompetitive antagonists of the P2X7 receptor. Several antagonists exhibited considerable species selectivity, which complicates their use across species and has obvious implications when developing P2X7 receptor antagonists for use in humans. Furthermore, pIC50 estimates could be affected by agonist concentration, temperature, and buffer composition to varying degrees. Given the wide range of conditions used for studying P2X7 receptors, together with the widespread use of pIC50 values to quantify antagonist potency, considerable caution must be considered when interpreting the results obtained with available antagonists.


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