Trinitrophenyl-Substituted Nucleotides Are Potent Antagonists Selective for P2X₁, P2X₃, and Heteromeric P2X₂/₃ Receptors

CATERINA VIRGINIO,¹ GRAEME ROBERTSON, ANNMARIE SURPRENANT, and R. ALAN NORTH
Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, Geneva, 1228 Switzerland (C.V., A.S., R.A.N.) and Exploratory Chemistry, Glaxo Wellcome Research and Development, Stevenage, SG1 2NY, United Kingdom (G.R.)

Received January 12, 1998; Accepted February 18, 1998 This paper is available online at http://www.molpharm.org

ABSTRACT
There are currently seven P2X receptor subunits (P2X₁₋₇) defined by molecular cloning. The functional identification of these receptors has relied primarily on the potency of ATP relative to that of ATP and on the kinetics of receptor desensitization. In the present experiments we found that the 2',3'-O-(2,4,6-trinitrophenyl)-substituted analogs of ATP are selective and potent antagonists at some but not all P2X receptors. The trinitrophenyl analogs of ATP, ADP, AMP, and GTP produced a reversible inhibition of ATP-evoked currents in human embryonic kidney 293 cells expressing P2X₁ receptors, P2X₃ receptors, or both P2X₂ and P2X₃ (heteromeric) receptors; IC₅₀ values were close to 1 nM. These compounds were at least 1000-fold less effective in blocking currents in cells expressing P2X₄, P2X₆, or P2X₇ receptors (P2X₅ and P2X₈ not tested). GTP, 2',4,6-trinitrophenol, and the 2',3'-trinitrophenyl analog of adenosine (0.1–10 μM) had no effect. Thus, we have identified a structural motif that confers antagonist action at P2X receptors that contain P2X₁ or P2X₃ subunits (the α,β-methylene-ATP-sensitive subclass).

There are seven P2X receptor subunits, which assemble into ATP-activated ion channels either as homomers or heteromers (reviewed by North, 1996; North and Barnard, 1997). At the molecular level, any pair of the subunits has 35–50% identical amino acids. At the functional level, several subgroups have been distinguished. For example, in one subgroup (P2X₁ and P2X₃ homomeric channels), αβmeATP and ATP are equally effective agonists, and the currents desensitize during agonist applications of more than several hundred milliseconds. None of the other homomeric channels is activated by αβmeATP, and the currents show much less desensitization. A distinct class of channel is formed by the coexpression of P2X₂ and P2X₃ subunits; this class is activated by αβmeATP and ATP but it shows little desensitization. A further distinguishing feature is the ability of PPADS to block the currents evoked by ATP; P2X₄, P2X₆, and P2X₇ receptors are relatively insensitive. Finally, P2X₃ homomeric channels are fundamentally different from all the others because repeated or prolonged agonist application results in cell permeabilization as measured by the uptake of fluorescent dyes and, eventually, cell lysis (North, 1996; Surprenant et al., 1996; North and Barnard, 1997).

The assignment of functional roles for P2X receptors in intact tissues depends critically on the use of receptor antagonists. Indeed, the main evidence that ATP mediates synaptic transmission between neurons (Edwards et al., 1992; Evans et al., 1992) or from nerve to muscle (Sneddon and Westfall, 1984; Evans and Surprenant, 1992) has been the block of the postsynaptic responses by suramin and/or PPADS (Sneddon and Westfall, 1984; Dunn and Blakey, 1988; Ziganshin et al., 1994). However, the low affinity and limited specificity of these compounds restricts their usefulness and, as mentioned above, some P2X receptors are not blocked (Buell et al., 1996). There is a clear need to identify more receptor antagonists.

Trinitrophenyl analogs of ATP have been widely used for the fluorescent labeling of ATP binding sites in proteins, and the trinitrophenyl analogs of ATP, ADP, AMP, and GTP produced a reversible inhibition of ATP-evoked currents in human embryonic kidney 293 cells expressing P2X₁ receptors, P2X₃ receptors, or both P2X₂ and P2X₃ (heteromeric) receptors; IC₅₀ values were close to 1 nM. These compounds were at least 1000-fold less effective in blocking currents in cells expressing P2X₄, P2X₆, or P2X₇ receptors (P2X₅ and P2X₈ not tested). GTP, 2',4,6-trinitrophenol, and the 2',3'-trinitrophenyl analog of adenosine (0.1–10 μM) had no effect. Thus, we have identified a structural motif that confers antagonist action at P2X receptors that contain P2X₁ or P2X₃ subunits (the α,β-methylene-ATP-sensitive subclass).

ABBREVIATIONS: αβmeATP, α,β-methylene-ATP; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPADS, pyridoxal 5-phosphate 6-azophenyl-2',4'-disulfonic acid; TNP, trinitrophenyl; TNP-A, 2',3'-O-(2,4,6-trinitrophenyl)-adenosine; TNP-ADP, 2',3'-O-(2,4,6-trinitrophenyl)-ADP; TNP-AMP, 2',3'-O-(2,4,6-trinitrophenyl)-AMP; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-ATP; TNP-GTP, 2',3'-O-(2,4,6-trinitrophenyl)-GTP; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

¹Current affiliation: Department of Pharmacology, Glaxo Wellcome Research and Development, 37135 Verona, Italy
including P2X receptors (Mockett et al., 1994). We first examined their effects on cloned and expressed P2X receptors with such an application in mind. In the course of those experiments, it became clear that, for some P2X receptors, the analogs were able to block responses to ATP at nanomolar concentrations. Here we report the characterization of this observation.

Experimental Procedures

HEK 293 cells that stably or transiently express the following P2X receptors were used in these studies: human P2X1, rat P2X2, rat P2X3, rat or human P2X4, rat P2X4 together with rat P2X4 (heteromer), and rat P2X7. Generation of stable P2X receptor-expressing cell lines and methods of transient lipofectin transfection have been described in detail previously (Evans et al., 1995; Buell et al., 1996; Evans et al., 1996; Kawashima et al., 1997). HEK cells stably transfected with the human P2X1 receptor were generously provided by Professor W. Stuhmer, Max-Planck Institute (Göttingen, Germany). Cells were plated onto 12-mm glass coverslips and maintained in Dulbecco’s modified Eagle’s medium, Nutrient Mix F-12 (GIBCO-BRL, Bethesda, MD) supplemented with 10% heat-inactivated fetal calf serum (FAKOLA, Bern, Switzerland) and 2 mM l-glutamine at 37° in a humidified 5% CO2 incubator.

Whole-cell recordings were made 12–48 hr after transient transfection (rat P2X1, P2X3, P2X4) and 6–72 hr after passage of stable cell lines (human P2X1, P2X3, P2X4, and rat P2X2, P2X2/3, and P2X7). Currents were recorded with an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany), acquired (1–2 kHz) and analyzed with Pulse and PulseFit 8.02 (HEKA). Patch pipettes (4–7 MΩ) contained 140 mM NaCl, 10 mM HEPES, and 11 mM...
EGTA. The external solution was 147 mM NaCl, 10 mM HEPES, 12 mM glucose, 2 mM KCl, 2 mM CaCl$_2$, and 1 mM MgCl$_2$. Osmolarity and pH values of both solutions were maintained at 300–315 mOsm/liter and 7.3, respectively. Unless otherwise stated, experiments were performed at a holding potential of ~60 mV and at room temperature. Agonists were applied using a flow of U-tube delivery system (Fenwick et al., 1982). Agonists were added to both the bath superfusate and the fast-flow solution. ATP was the agonist in all experiments on P2X$_1$, P2X$_{2/3}$, P2X$_4$, and P2X$_7$ receptors. Both ATP and αβmeATP were used at the P2X$_3$ receptor and only αβmeATP was used at the heteromeric P2X$_{2/3}$ receptor (Kawashima et al., 1997). Agonists were applied for 0.5–2-sec duration at 2 min intervals at all receptors except P2X$_1$ and P2X$_3$ where 4–5 min intervals were required to allow recovery from desensitization (Evans et al., 1995).

Antagonist concentration-response curves for each cell were fit by the least-squares method to $I = I_{\text{max}}[1 + (\text{EC}_{50}/[A])^{nH}]$ where $I$ is the peak current evoked by agonist concentration [A], $I_{\text{max}}$ is the peak current evoked by a maximal agonist concentration, EC$_{50}$ is the concentration giving half the maximal current, and $n_H$ is the Hill coefficient. Antagonist concentration-inhibition curves were obtained in individual cells by using a fixed agonist concentration close to the EC$_{50}$ (1 μM ATP at P2X$_1$, 10 μM ATP at P2X$_2$ and P2X$_4$, 300 μM ATP at P2X$_3$, 1 μM ATP or αβmeATP at P2X$_7$, and 5 μM αβmeATP at P2X$_{2/3}$), and progressively increasing the concentration of antagonist: IC$_{50}$ values were calculated by least squares fitting to the logistic function (see Experimental Procedures); the IC$_{50}$ values given in text and tables are the mean ± standard error from 4–8 experiments. 

TABLE 1

Inhibition of currents evoked by ATP or αβmeATP in cells expressing P2X receptor

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X$_1$</td>
<td>6 ± 3 (6)</td>
</tr>
<tr>
<td>P2X$_3$</td>
<td>0.9 ± 0.2 (6)</td>
</tr>
<tr>
<td>P2X$_{2/3}$</td>
<td>7 ± 3 (5)</td>
</tr>
<tr>
<td>P2X$_2$</td>
<td>2,000 ± 200 (7)</td>
</tr>
<tr>
<td>P2X$_4$</td>
<td>15,200 ± 410 (6)</td>
</tr>
<tr>
<td>P2X$_7$</td>
<td>&gt;30,000 (6)</td>
</tr>
</tbody>
</table>

Results

TNP-AMP strongly inhibited currents in cells expressing P2X$_1$, P2X$_3$, or P2X$_{2/3}$ receptors (IC$_{50}$ about 1 nM), but was very much less effective in cells expressing P2X$_2$, P2X$_4$, or P2X$_7$ receptors (IC$_{50}$ > 1 μM) (Figs. 1 and 2, Table 1). The inhibition was concentration-dependent and well fitted by the logistic function (see Experimental Procedures); the IC$_{50}$ values are shown in Table 1, and the coefficient $n_H$ was not significantly different from unity. The inhibition reversed within 4–15 min of TNP-AMP washout, although reversal...
was sometimes incomplete for near maximal concentrations. The inhibition was the same at holding potentials of -60 mV and 40 mV (n = 4, 6, 5, and 3 for cells expressing P2X3, P2X2/3, P2X2, and P2X4 receptors, respectively). The P2X5 receptor was particularly insensitive to blocking by TNP-ATP; at the highest concentration tested (30 μM), the inhibition was 39 ± 2% (n = 6). TNP-ATP (1 nm-30 μM) had no agonist action at any of the P2X receptors.

The effect of TNP-ATP was mimicked by TNP-ADP and TNP-AMP, as well as TNP-GTP (Fig. 3), although TNP-A had no effect (n = 4). These compounds were also highly effective at P2X1, P2X3, and P2X2/3 receptors but much less so at P2X2, P2X4, and P2X7 receptors. Complete antagonist-inhibition curves were generated for P2X3, P2X5, and P2X2/3 receptors (Fig. 3), and IC50 values are provided in Table 1. The dose-inhibition curves at the heteromeric P2X2/3 receptors were consistently to the right of those for the homomeric P2X3 receptor (Fig. 3), although the difference in IC50 estimates was significant only in the case of TNP-ATP (Table 1). As for TNP-ATP, the other TNP-nucleotides (up to 30 μM) had no agonist action. TNP-A (0.1–1 μM), GTP (0.1–10 μM), and picric acid (10 μM) had no agonist or antagonist action at P2X3, P2X2/3, or P2X5 receptors.

The nature of the inhibition was examined further in the case of the P2X3 receptor by constructing full agonist concentration-response curves. With either ATP or αβmeATP as the agonist, TNP-ATP (3 and 10 nm, respectively) caused both a rightward shift and a depression of the maximal current, indicating insurmountable antagonism. For the two antagonist concentrations ([B]/3 and 10 nm, respectively), the curves were fit by an expression appropriate to noncompetitive antagonism ([I]max = [1 + (EC50/[A]) + 1 + Kf/[B])−1], which provided estimates of Kf of about 2 nm. Similar results were obtained for TNP-ADP and TNP-GTP (data not shown).

### Discussion

The results indicate that certain nucleotides with a ribose-substituted trinitrophenyl group are potent antagonists at those P2X receptors that can be activated by αβmeATP (P2X1, P2X4, and P2X2/3). However, several observations are not readily reconciled with the notion that the TNP-nucleotides are binding to the site occupied by the agonists when they act to open the P2X receptor channel. First, the antagonism is noncompetitive (Fig. 4). Second, both guanine and adenine nucleotides are equally effective; this is in marked contrast to the lack of any agonist activity by GTP itself. Third, removal of one or even two phosphate groups from TNP-ATP had no significant effect on the antagonism; yet in terms of agonist action, ADP is more than 100-fold less potent than ATP at the P2X3 receptor (Lewis et al., 1995) and AMP (100 μM) has no effect at the P2X7 (Evans et al., 1995), P2X3 (Chen et al., 1995) or P2X2/3 receptor (unpublished observations). Removal of the third phosphate, as in TNP-A, resulted in loss of antagonism. In brief, the antagonist binding site differs from the agonist binding site in that it does not discriminate between guanine or adenine bases, absolutely requires the 2',3'-trinitrophenol, and will accept one, two, or three (but not zero) 5'-phosphates.

It is possible that the TNP-nucleotides directly block the conducting pathway of the channel, as found for the outwardly rectifying chloride channel (Paulmichi et al., 1992; Venglark et al., 1993). This seems unlikely both because ATP is negatively charged and the channel is cation-selective, and because the inhibition by TNP was not different for inward and outward currents. In any event the concentrations of extracellular ATP and TNP-ATP that block the outwardly rectifying chloride current are still some hundred-fold higher than those effective at P2X1, P2X3, and P2X2/3 receptors. The most likely mechanism, therefore, is the binding of TNP-nucleotides to an allosteric site on the large extracellular region of the receptor. In this case, the P2X3 and P2X5 subunits might provide a common domain that interacts with the strongly electronegative trinitrophenyl moiety. It is interesting that the most potent antagonists in a series tested on the rat urinary bladder (which expresses P2X4 receptors) also had large aromatic 3' substitutions (Bo et al., 1994; Burnstock et al., 1994); these bound with affinities in the 10–100 nm range.

The weak antagonism of TNP-ATP at other receptors had previously been reported for cochlear hair cells isolated from guinea pig organ of Corti (Mockett et al., 1994), which are known to express P2X2 receptors (Housley et al., 1995; Brandle et al., 1997). In that case, 75 μM TNP-ATP almost completely blocked the current evoked by 10 μM ATP. TNP-ATP is not an effective antagonist in the rat parotid gland.

---

**Fig. 4.** TNP-ATP is a noncompetitive antagonist at the P2X3 receptor. a. Currents recorded from a single cell expressing the P2X3 receptor in the absence (top) and presence (bottom) of 10 nM TNP-ATP. b and c. Agonist concentration-response curves for αβmeATP (b) and ATP (c) in the absence and presence of 3 nM or 10 nM TNP-ATP. Points, mean ± standard error of 8–9 experiments for αβmeATP and 3–6 experiments for ATP.
(Soltoff et al., 1993), which contains P2X4 (Buell et al., 1996) and P2X7 receptors (Collo et al., 1997). It will clearly be important to test the TNP analogs on P2X responses to ATP in other tissues. On the basis of the present work, one might expect blockade in the nanomolar concentration range to indicate that the underlying receptor contains P2X4 or P2X3 subunits. The results with heteromeric receptors, such as are expressed by some primary afferent neurons (Lewis et al., 1995; Cook et al., 1997), might be less straightforward. In the present work, we used αβmεATP as the agonist in experiments on the cells expressing the heteromeric P2X2/3 receptor, on the assumption that it activates only heteromers and not any homomeric P2X3 receptors that might also be present. The nerve-released transmitter would be ATP rather than αβmεATP, and a combined action of P2X2 and P2X3 subunits might result in an intermediate sensitivity to TNP-ATP. For the interpretation of such experiments it would also be useful to know whether these TNP analogs have blocking action at other receptors types, including the P2Y receptors.

Acknowledgments

We thank D. Estoppey for her skilled assistance with cell culturing.

References


