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Pharmacological characterization of recombinant human and rat P2X receptor subtypes

Bruce R. Bianchi, Kevin J. Lynch, Edward Touma, Wende Niforatos, Edward C. Burgard, Karen M. Alexander, Helen S. Park, Haixia Yu, Randy Metzger, Elizabeth Kowaluk, Michael F. Jarvis, Tim van Biesen *

Neurological and Urological Diseases Research, Pharmaceutical Products Division, Abbott Laboratories, D-4PM, AP10, 100 Abbott Park Rd., Abbott Park, IL 60064-3500, USA

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Abstract

ATP functions as a fast neurotransmitter through the specific activation of a family of ligand-gated ion channels termed P2X receptors. In this report, six distinct recombinant P2X receptor subtypes were pharmacologically characterized in a heterologous expression system devoid of endogenous P2 receptor activity. cDNAs encoding four human P2X receptor subtypes ($hP2X_1$, $hP2X_3$, $hP2X_4$, and $hP2X_7$), and two rat P2X receptor subtypes ($rP2X_2$ and $rP2X_3$), were stably expressed in 1321N1 human astrocytoma cells. Furthermore, the $rP2X_2$ and $rP2X_3$ receptor subtypes were co-expressed in these same cells to form heteromultimeric receptors. Pharmacological profiles were determined for each receptor subtype, based on the activity of putative P2 ligands to stimulate Ca^{2+} influx. The observed potency and kinetics of each response was receptor subtype-specific and correlated with their respective electrophysiological properties. Each receptor subtype exhibited a distinct pharmacological profile, based on its respective sensitivity to nucleotide analogs, diadenosine polyphosphates and putative P2 receptor antagonists. αβ-methylene ATP (αβ-meATP), a putative P2X receptor-selective agonist, was found to exhibit potent agonist activity only at the $hP2X_1$, $hP2X_3$ and $rP2X_3$ receptor subtypes. Benzoylbenzoic ATP (BzATP, 2′ and 3′ mixed isomers), which has been reported to act as a $P2X_7$ receptor-selective agonist, was least active at the rat and human $P2X_7$ receptors, but was a potent (nM) agonist at $hP2X_1$, $rP2X_3$ and $hP2X_3$ receptors. These data comprise a systematic examination of the functional pharmacology of P2X receptor activation.

Keywords: ATP; P2X receptor; Purinergic; Recombinant

1. Introduction

The proposed role of ATP as a functional neurotransmitter and local intercellular signaling molecule (Burnstock, 1972) has gained widespread acceptance since the cloning of cell-surface receptors specifically activated by purine and pyrimidine nucleotides (P2 receptors) (Burnstock, 1996; Williams and Burnstock, 1997). These receptors are sub-classified into two broad groups, P2X and P2Y, based on their structural and functional similarities (Burnstock and Kennedy, 1985; Fredholm et al., 1997). P2X receptors are multimeric ligand-gated ionotropic channels that exhibit a non-selective cation permeability, and share a common structural motif characterized by two transmembrane spanning domains connected by an extracellular loop. P2Y receptors are members of the G protein-coupled receptor family (metabotropic receptors), characterized by seven transmembrane spanning domains and a signaling mechanism dependent on heterotrimeric G proteins (Harden et al., 1995).

To date, seven functional P2X and five functional P2Y receptors have been identified by molecular cloning techniques. P2Y receptors are activated by a variety of nucleotide ligands, including purine and pyrimidine nucleotides both in the diphosphate and triphosphate forms (Harden et al., 1995; Communi and Boeynaems, 1997). In contrast, P2X receptors appear to share ATP as their endogenous ligand and may exhibit signaling specificity based primarily on differential tissue localization, agonist sensitivity, and quaternary structure (i.e., homo- vs. heteromultimerization) (Williams and Burnstock, 1997). In addition to the P2X and P2Y receptor families, additional

* Corresponding author

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nucleotide receptors have been postulated on the basis of radioligand binding assays and functional activity. However, these novel receptor subtypes, including P2T (activated by ADP) (Hourani and Hall, 1996) and P2D (activated by diadenosine polyphosphates) (Miras-Portugal et al., 1996) receptors, have not yet been identified at the molecular level.

The recombinant $P2X_1$ receptor has been expressed transiently in *Xenopus* oocytes and HEK293 cells, where it exhibits sensitivity to the agonist αβ-methylene ATP (αβ-meATP) and rapid desensitization kinetics (Valera et al., 1994). In contrast, the $P2X_2$ receptor, originally cloned from rat PC12 cells, is insensitive to αβ-meATP and desensitizes slowly after agonist activation (Brake et al., 1994; Evans et al., 1995). Both the $P2X_1$ and $P2X_2$ receptor subtypes are sensitive to inhibition by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and suramin. The pharmacological and kinetic properties of the $P2X_3$ receptor from rat dorsal root ganglia (Chen et al., 1995; Lewis et al., 1995) and human heart (Garcia-Guzman et al., 1997b) are similar to those reported for the $P2X_1$ receptor, including αβ-meATP sensitivity and rapid desensitization kinetics. The $P2X_4$ receptor is non-desensitizing, αβ-meATP sensitive and has been identified in rat hippocampus (Bo et al., 1995), rat superior cervical ganglion (Buell et al., 1996) and human brain (Garcia-Guzman et al., 1997a). The rat and human homologs of the $P2X_4$ receptor appear to differ in their sensitivity to suramin and PPADS, where the human $P2X_4$ receptor is weakly sensitive and the rat $P2X_4$ receptor is insensitive to these putative inhibitors (Garcia-Guzman et al., 1997a). The $P2X_7$ receptor is distinguished by its ability to form a large pore upon prolonged or repeated agonist stimulation (Rassendren et al., 1997). $P2X_7$ is partially activated by saturating concentrations of ATP, whereas it is fully activated by the synthetic ATP analog benzoylbenzoic ATP (BzATP, 2′ and 3′ mixed isomers) (Gargett et al., 1997).

The $P2X_2$ and $P2X_3$ receptor subtypes have been shown to form functional heteromeric receptors in native tissues (Lewis et al., 1995). The $P2X_{2/3}$ heteromeric receptor appears to combine the pharmacological properties of $P2X_3$ (αβ-meATP sensitivity) with the kinetic properties of $P2X_2$ (slow desensitization) (Lewis et al., 1995; Radford et al., 1997), thereby facilitating its detection in situ or in heterologous expression systems.

To date, two additional P2X receptor subtypes have been identified, $P2X_5$ and $P2X_6$ (Collo et al., 1996; Garcia-Guzman et al., 1996). However, the $P2X_5$ receptor mediates a very weak response to nucleotide agonists such that receptor-mediated changes in Ca^{2+} influx cannot be reliably measured. In contrast, the $P2X_6$ receptor mediates a potent response to ATP, but is poorly expressed in heterologous cell systems (Collo et al., 1996). In vivo, these receptors may not exist as homomeric receptors, but rather as heteromers with other P2X receptor subtypes. Recently, the $P2X_4$ and $P2X_6$ receptor subtypes have been shown to form functional heteromers in vitro (Le et al., 1998).

Attempts to characterize endogenous and recombinant P2X receptor subtypes using radioligand binding techniques have met with limited success, primarily due to a lack of selective radioligands. Recent reports have called into question the binding specificity of a number of putative P2 receptor-selective radioligands, including [^{35}S]-deoxyadenosine 5′-*O*-(1-thiotriphosphate) (dATPαS) (Schachter and Harden, 1997), [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), and [^{35}S]adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) (Bianchi et al., 1998). These reports reveal no correlation between radioligand binding profiles and the functional activity of various P2 ligands, and thus conclude that radiolabeled nucleotide analogs are unsuitable for identifying and discriminating between P2 receptor subtypes expressed at physiological levels. The lack of P2 receptor subtype-selective ligands has complicated the characterization of these receptors in situ.

To provide a direct comparative pharmacological characterization of the P2X receptors, we have cloned the rat $P2X_2$ and $P2X_3$ receptors, and the human $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ receptors. Each P2X receptor subtype was functionally expressed in its homomeric form in stably transfected 1321N1 human astrocytoma cells, which have previously been shown to be devoid of endogenous P2X or P2Y receptor function (Bianchi et al., 1998). The rat $P2X_2$ and $P2X_3$ receptor subunits were co-expressed to form the functional $P2X_{2/3}$ heteromeric receptor. The functional and pharmacological properties of P2X receptor-mediated Ca^{2+} influx were characterized in each cell line using a fluorescence based Ca^{2+} influx assay. The resulting pharmacological profiles were unique to each cell line and serve as a useful tool in the identification and characterization of endogenously expressed P2X receptors, while further emphasizing the need for P2 receptor subtype-selective ligands.

2. Materials and methods

2.1. Materials

ATP, 2-methylthio-ATP (2-meS-ATP), αβ-meATP, suramin, and PPADS were obtained from Research Biochemicals International (Natick, MA). BzATP, ATPγS, ADP, UTP, and diadenosine polyphosphates (AP_nA, where $n = 3–6$) were obtained from Sigma (St. Louis, MO). ADPβS and G418 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Dulbecco's modified Eagle's medium (D-MEM) (with 4.5 mg ml^{-1} glucose and 4 mM L-glutamine) and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT). Phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (D-PBS) (with 1 mg ml^{-1} glucose and 3.6 mg l^{-1} Na pyruvate, without phenol red), hygromycin B and Lipofec-

tamine were obtained from Life Technologies (Grand Island, NY). Fluo-3 AM (*N*-[4-[6-[(acetyloxy)methoxy]-2,7-dichloro-3-oxo-3*H*-xanthen-9-yl]-2-[2-[bis[2-[(acetyloxy) methoxy] - 2 - oxyethyl] amino] - 5 - methylphenoxy] ethoxy] phenyl] *N* - [2 - [(acetyloxy) methoxy] - 2 - oxyethyl] glycine, (acetyloxy)methyl ester) and YO-PRO-1 (4-[(3-methyl-2(3*H*)-benzoxazolylidene) methyl]-1-[3-(trimethylammonio) propyl]-quinolinium, diiodide) were purchased from Molecular Probes (Eugene, OR).

2.2. Cloning and expression of P2X receptor subtypes

Clones containing the human $P2X_4$ and $P2X_7$ receptor cDNAs were obtained from Incyte Pharmaceuticals (Palo Alto, CA). The cDNAs for the human $P2X_1$ and $P2X_3$, and rat $P2X_2$ and $P2X_3$, receptors were isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) cloning from various poly A^+ RNAs supplied by Clontech (Palo Alto, CA) using primers designed surrounding the initiation and termination codons of the published sequences (Genbank accession numbers: human $P2X_1$, X83688; human $P2X_3$, Y07683; rat $P2X_2$, U14114; rat $P2X_3$, X90651).

The resulting cDNA PCR products were subcloned into either the pCRII vector (Invitrogen, Carlsbad, CA) or pCRscript vector (Stratagene, La Jolla, CA) and fully sequenced using fluorescent dye-terminator reagents (Prism, Perkin-Elmer Applied Biosystems Division) and a Perkin-Elmer Applied Biosystems Model 373 DNA sequencer or Model 310 genetic analyzer. All messages were found to encode polypeptides identical to the published sequences with the exception of the human $P2X_3$ receptor cDNA. The human $P2X_3$ cDNA was identical to that reported by Garcia-Guzman et al. (1997b) except for an arginine at amino acid residue 126; the published sequence encodes a proline at this position. The predicted polypeptide sequence of the human $P2X_4$ receptor sequence was found to be identical to the published human P2X4 sequence (Garcia-Guzman et al., 1997a) with the exception of two residues; at residue 6 an alanine replaces a serine of the published sequence and residue 242 a glycine replaces a serine. Several independent clones were sequenced to control for mutations introduced via PCR.

The human $P2X_1$, $P2X_3$, and $P2X_4$ cDNAs were transferred to the mammalian expression vector pCDNA3.1(neo) (Stratagene). The vector pIRES(hyg) (Clontech) was employed for rat $P2X_2$ expression, and pIRES(neo) (Clontech) was used to express the rat $P2X_3$ and human $P2X_7$ cDNAs.

Expression plasmids encoding each P2X receptor cDNA were transfected individually into 1321N1 human astrocytoma cells using Lipofectamine. Forty-eight hours after transfection, cells were subcultured in growth medium containing 800 $\mu g\ ml^{-1}$ G418 ($hP2X_1$, $rP2X_3$, $hP2X_3$, $hP2X_4$, and $hP2X_7$) or 100 $\mu g\ ml^{-1}$ hygromycin B ($rP2X_2$). Surviving individual colonies were isolated and screened for P2 receptor activity. The clones exhibiting the largest ATP-induced response were selected for further characterization and given the following designations: $1321hX_1$-39 ($hP2X_1$), $1321rX_2$-1 ($rP2X_2$), $1321rX_3$-3 ($rP2X_3$), $1321hX_3$-11 ($hP2X_3$), $1321hX_4$-15 ($hP2X_4$) and $1321hX_7$-1 ($hP2X_7$). The cell line expressing heteromeric $rP2X_{2/3}$ ($1321rX_{2/3}$-2) receptors was constructed by transfection of the $rP2X_3$ cDNA into $1321rX_2$-1 cells. Positive clones were isolated in growth medium containing 150 $\mu g\ ml^{-1}$ G418 and 75 $\mu g\ ml^{-1}$ hygromycin B and selected based on their sensitivity to $\alpha\beta$-meATP.

Cells expressing recombinant homomeric P2X receptors were maintained at 37°C in D-MEM (with 4.5 mg ml^{-1} glucose and 4 mM L-glutamine), 10% fetal bovine serum and 300 $\mu g\ ml^{-1}$ G418 or 100 $\mu g\ ml^{-1}$ hygromycin B in a humidified 5% CO_2 atmosphere. $1321rX_{2/3}$-2 cells were maintained in growth medium containing 150 $\mu g\ ml^{-1}$ G418 and 75 $\mu g\ ml^{-1}$ hygromycin B.

2.3. Measurement of intracellular Ca^{2+} levels

P2X receptor function was determined on the basis of agonist-mediated increases in cytosolic Ca^{2+} concentration. The fluorescent Ca^{2+} chelating dye fluo-3 was used as an indicator of the relative levels of intracellular Ca^{2+} in a 96-well format using a Fluorescence Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA). Cells were grown to confluence in 96-well black-walled tissue culture plates and loaded with fluo-3 AM (2 μM) in D-PBS for 1–2 h at 23°C. Prior to the assay, each plate was washed three times with 250 μl D-PBS to remove extracellular fluo-3 AM. All compound solutions were prepared in D-PBS. Fluorescence data were collected for 3 min following the addition of agonists (50 μl of 4 $\times$ concentration) (final volume = 200 μl/well). Antagonists (50 μl of 4 $\times$ concentration) were added 3 min before the addition of agonists (50 μl of 4 $\times$ concentration) (final volume = 200 μl). Ligands were tested at 11 concentrations (indicated on each graph). Fluorescence data was collected at 1- to 5-s intervals throughout each experimental run.

Data shown are based on the peak increase in relative fluorescence units as compared to basal fluorescence. Concentration-effect curves for all cell types, except $1321rX_7$-11, are shown as a percentage of the maximum ATP-mediated signal. For $1321rX_7$-11 cells, BzATP was used to define the maximum signal. Dose response data were analyzed using a four-parameter logistic Hill equation in GraphPad Prism (San Diego, CA).

2.4. Measurement of pore formation in $1321hX_7$-11 cells

Formation of large membrane pores by $hP2X_7$ receptors was measured as a function of permeability to the

fluorescent propidium dye YO-PRO-1 using methods previously described (Rassendren et al., 1997). Cells were grown to confluence and the cell culture medium was replaced with PBS containing 2 μM YO-PRO-1 prior to the assay. A 4× final concentration of BzATP was prepared in 2 μM YO-PRO-1 and 50 μl was added to the cells to start the experimental run (final volume = 200 μl/well). Fluorescence data were collected at 10 s intervals over a 20 min period using FLIPR.

2.5. Electrophysiology

All P2 receptor clones were initially characterized in a *Xenopus* oocyte expression system. The $rP2X_2$, $hP2X_3$, $rP2X_{2/3}$ and $hP2X_4$ receptor subtypes expressed in 1321N1 cells were further characterized using conventional whole-cell patch-clamp recording techniques. Briefly, cells were maintained in an extracellular recording solution (pH 7.4, 325 mosM) consisting of (mM): 155 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 12 glucose, 10 HEPES. Recording pipettes were filled with an internal solution (pH 7.3, 295 mosM) consisting of (mM): 140 K-aspartate, 20 NaCl, 10 EGTA, 5 HEPES. Agonist was rapidly applied using a piezoelectric-driven theta tube. No differences were observed in either the pharmacological or kinetic properties of the P2 receptor clones between the oocyte and 1321N1 expression systems. For analysis of $hP2X_1$ and $hP2X_7$ receptors, intranuclear injections of 12 nl cDNA (1 μg/μl) were performed on defolliculated *Xenopus* oocytes. Receptor expression was analyzed 3–5 days later using two-electrode voltage-clamp techniques. Oocytes were perfused with a standard recording solution containing (mM): 96 NaCl, 2.0 KCl, 1.8 $BaCl_2$, 1.0 $MgCl_2$, 5.0 Na-pyruvate, and 5.0 Na-HEPES (pH 7.4), and intracellular electrodes were filled with 120 mM KCl. Agonist was applied using a small diameter tube directed at the cell. For all experiments, cells were voltage-clamped at −60 mV, and data was acquired and analyzed using pClamp software (Axon Instruments, Foster City, CA). At least three cells were analyzed for each receptor subtype expressed.

3. Results

3.1. Kinetics of ATP-activated P2X receptor responses

Activation of each P2X receptor subtype leads to a rapid increase in cytosolic Ca^{2+} levels (Fig. 1). The kinetics of Ca^{2+} influx is reflective of the electrophysiological response for each P2X receptor subtype (Fig. 2). The kinetics of the ligand-activated transmembrane currents, in turn, are consistent with previously reported data (see references in Section 1). Briefly, $hP2X_1$ and $hP2X_3$ receptors exhibit rapid desensitization kinetics whereas $rP2X_2$, $hP2X_4$, $rP2X_{2/3}$ and $hP2X_7$ desensitize slowly, if at all (Fig. 2).

The heteromeric $rP2X_{2/3}$ receptor has previously been shown to reflect the pharmacological properties of the homomeric $rP2X_3$ receptor and the kinetic properties of the homomeric $rP2X_2$ receptor (Lewis et al., 1995). Consistent with this report, $1321rX_{2/3}$-2 cells are characterized by their sensitivity to αβ-meATP and by a non-desensitizing response (Fig. 1DFig. 2D). Activation of $1321rX_{2/3}$-2 cells with ATP leads to a larger Ca^{2+} influx than is mediated by αβ-meATP, suggesting the presence of homomeric $rP2X_2$ receptors in these cells. αβ-meATP elicited a non-desensitizing response (Fig. 1D) similar to that mediated by ATP activation of the homomeric $rP2X_2$ receptor (Fig. 1B).

The $hP2X_7$ receptor is a non-desensitizing receptor unique in its ability to form a transmembrane pore permeable to molecules up to 900 Da (Fig. 2F) (Steinberg et al., 1987). Activation of $1321hX_7$-1 cells by BzATP mediates a potent Ca^{2+} influx which remains relatively constant at its maximal level (Fig. 1F). Rather than reflecting the desensitization properties of the receptor, this effect is more likely due to the efflux of fluo-3 through the $hP2X_7$ receptor-mediated pore into the extracellular medium (Rassendren et al., 1997). The rapid formation of a transmembrane pore in response to $hP2X_7$ receptor activation is confirmed by the onset of membrane permeabilization as determined by the influx of YO-PRO-1 (MW = 650) into $1321hX_7$-1 cells following receptor activation by BzATP (Fig. 1F, inset).

The absolute magnitude of the Ca^{2+} influx is likely to be a function of the level of receptor expression, channel activation and desensitization kinetics, and the transmembrane driving force. Thus, in the absence of a reliable radioligand binding assay to quantitate receptor expression, the relative magnitudes of the responses mediated by various stably transfected cell lines cannot be directly compared.

3.2. Functional pharmacology of P2X receptor subtypes

Fig. 3 shows concentration-effect curves for prototypic P2 receptor agonists assayed at all of the P2X receptor-expressing cell lines described above. Fig. 4 shows the activity of the putative P2 receptor antagonists, suramin and PPADS. pEC_{50} and pIC_{50} values for these data, as well as for various diadenosine polyphosphates, are summarized in Table 1.

The $hP2X_1$ receptor was activated by submicromolar concentrations of several purine nucleotide agonists, including ATP, 2-meS-ATP and αβ-meATP (Table 1, Fig. 3A). BzATP, which has previously been described as a $P2X_7$-selective agonist (Wiley et al., 1996), was shown to be the most potent $hP2X_1$ agonist ($pEC_{50} = 8.74$). In

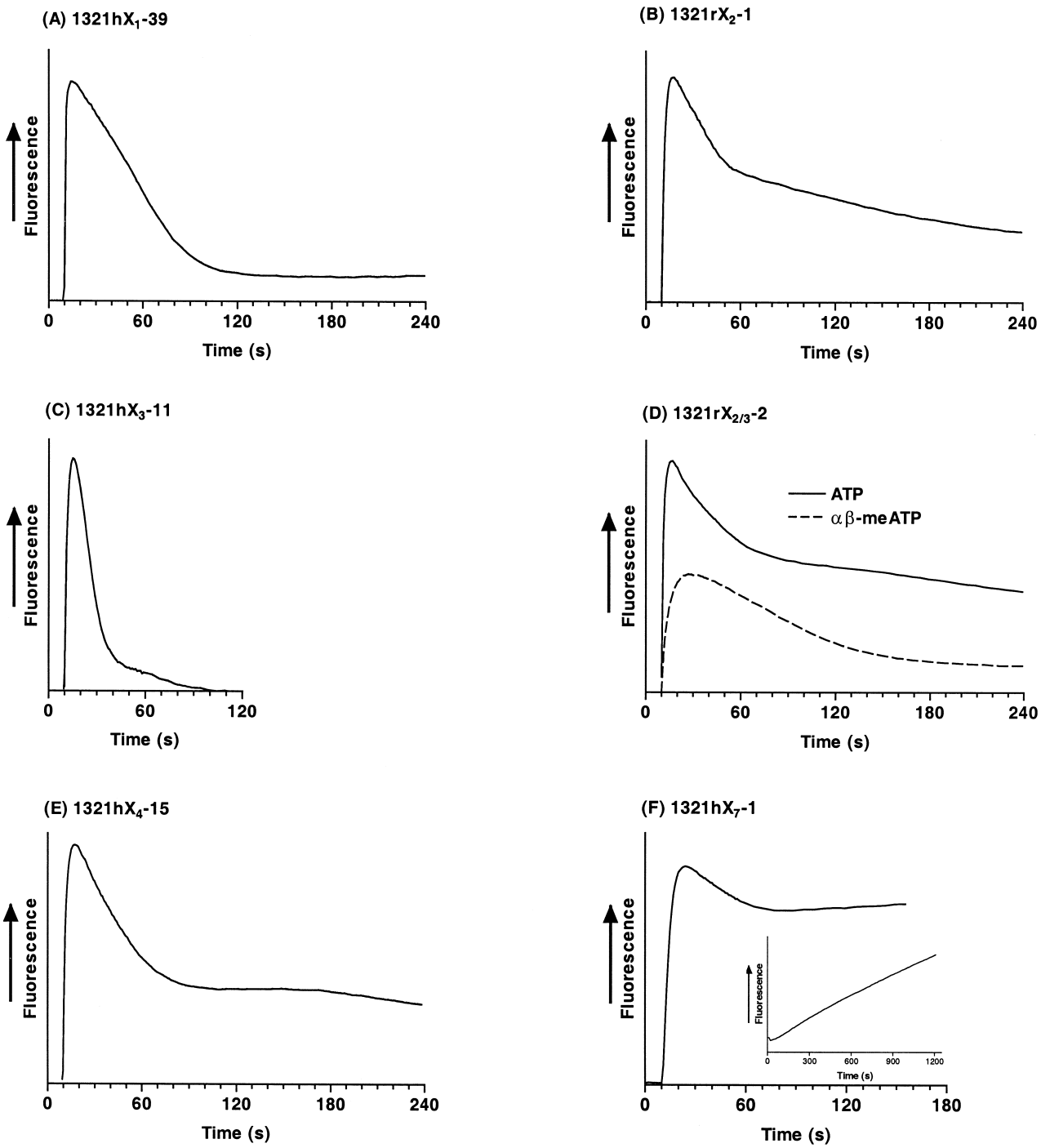

Fig. 1. Kinetics of ATP-activated Ca^{2+} influx into cells expressing P2X receptor subtypes. Each of the cell lines shown was activated by the following agonists: (A) 1321hX_1-39 (0.5 μM ATP), (B) 1321rX_2-1 (10 μM ATP), (C) 1321hX_3-11 (3 μM ATP), (D) 1321r$X_{2/3}$-2 (10 μM ATP or 10 μM αβ-meATP), (E) 1321hX_4-15 (4 μM ATP), (F) 1321hX_7-1 (25 μM BzATP). (F) *inset*: Kinetics of BzATP-activated YO-PRO-1 influx. Agonists were added 10 s after initiation of the experimental run.

addition to purine nucleotides, the diadenosine polyphosphates AP_4A and AP_5A activated the h$P2X_1$ receptor at submicromolar concentrations (Table 1). AP_6A functioned as a partial agonist while AP_2A and AP_3A were inactive as agonists at the h$P2X_1$ receptor. h$P2X_1$ receptor activation was fully blocked by comparable concentrations of either suramin or PPADS (pIC_{50} = 6.07 and 5.52, respectively) (Fig. 4A, Table 1).

The r$P2X_2$ receptor was maximally activated by ATP, 2-meS-ATP, ATPγS and BzATP (Fig. 3B). However, as has previously been reported, αβ-meATP was inactive as an agonist at this receptor (Simon et al., 1997) (Table 1).

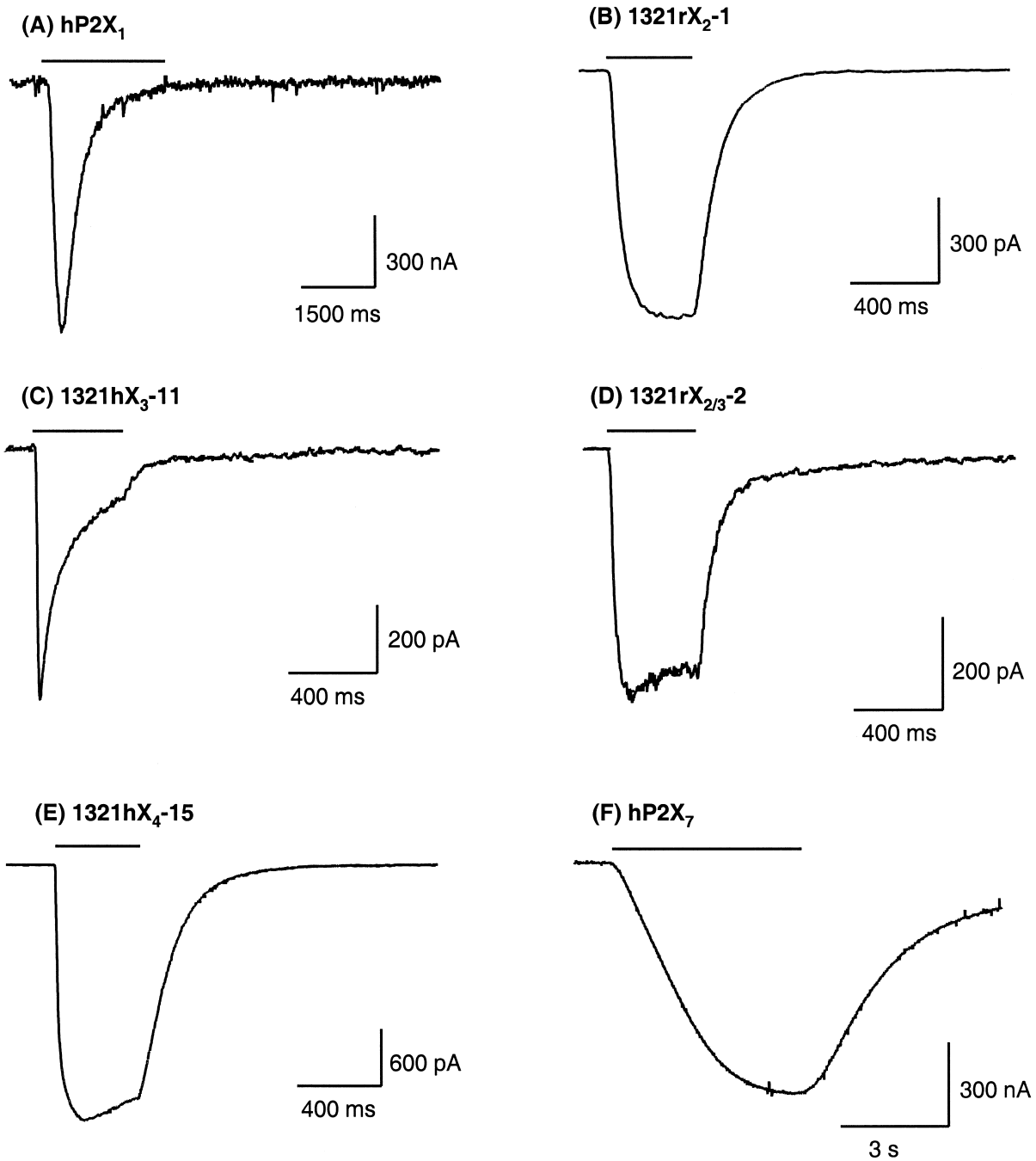

Fig. 2. Electrophysiological responses to P2X receptor activation. Kinetics of $hP2X_1$ and $hP2X_7$ receptors were measured in cDNA-injected *Xenopus* oocytes. $rP2X_2$, $hP2X_3$, $rP2X_{2/3}$ and $hP2X_4$ receptors were assayed in their corresponding 1321N1 stable cell lines. The duration of agonist application is shown by the horizontal bar. Receptors were activated as follows: (A) $hP2X_1$ (10 μM ATP), (B) $1321rX_2$-1 (10 μM ATP), (C) $1321hX_3$-11 (10 μM ATP), (D) $1321rX_{2/3}$-2 (10 μM αβ-meATP), (E) $1321hX_4$-15 (100 μM ATP), (F) $hP2X_7$ (100 μM BzATP). Cells were voltage-clamped at −60 mV.

The $rP2X_2$ receptor described here represents the full-length clone ($rP2X_{2a}$ form). A previously identified splice variant of the $rP2X_2$ receptor ($rP2X_{2b}$) (Simon et al., 1997), which carries a deletion in the C-terminus and is reported to exhibit altered desensitization kinetics (Koshimizu et al., 1998), was pharmacologically identical to the receptor shown here (data not shown). The $rP2X_2$ receptor was insensitive to activation by diadenosine polyphosphates (Table 1). PPADS ($pIC_{50} = 5.42$) was 10-fold more potent than suramin ($pIC_{50} = 4.48$) as a $rP2X_2$ receptor antagonist (Fig. 4B, Table 1).

Cells expressing either the rat or human $P2X_3$ receptor ($1321rX_3$-3 and $1321hX_3$-11 cells, respectively), were slightly more sensitive to purine nucleotides when com-

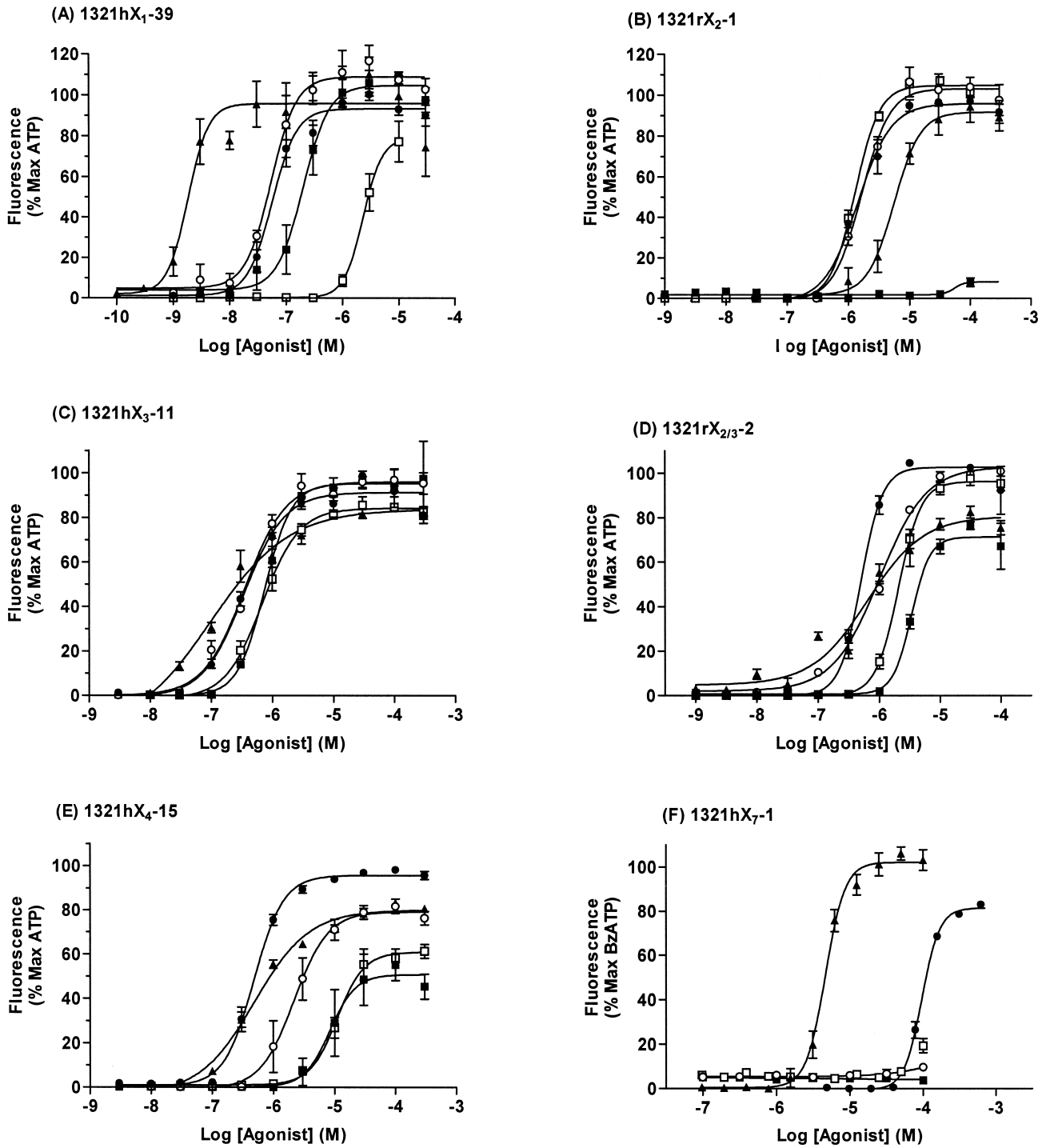

Fig. 3. Functional characterization of P2X receptor agonists. P2X receptor function was measured using a fluo-3 Ca^{2+} influx assay. Each of the cell lines shown was treated with increasing concentrations of ATP (●), 2-meS-ATP (○), αβ-meATP (■), ATPγS (□), or BzATP (▲). Data are shown as the mean percentage of the maximum signal mediated by ATP (or BzATP, (F) ±S.E.M. ($n = 3$).

pared to the $rP2X_2$ receptor. However, the $P2X_3$ receptor was functionally distinguished from the $P2X_2$ receptor by its submicromolar sensitivity to αβ-meATP (Fig. 3C, Table 1). Like the $hP2X_1$ receptor, the rat and human $P2X_3$ receptors were fully activated by low concentrations of BzATP (Fig. 3C, Table 1). The $hP2X_3$ receptor was sensitive to activation by submicromolar concentrations of AP_4A, AP_5A and AP_6A, although all responses mediated by diadenosine polyphosphates were submaximal (Table 1). Suramin exhibited submaximal inhibition of the $hP2X_3$

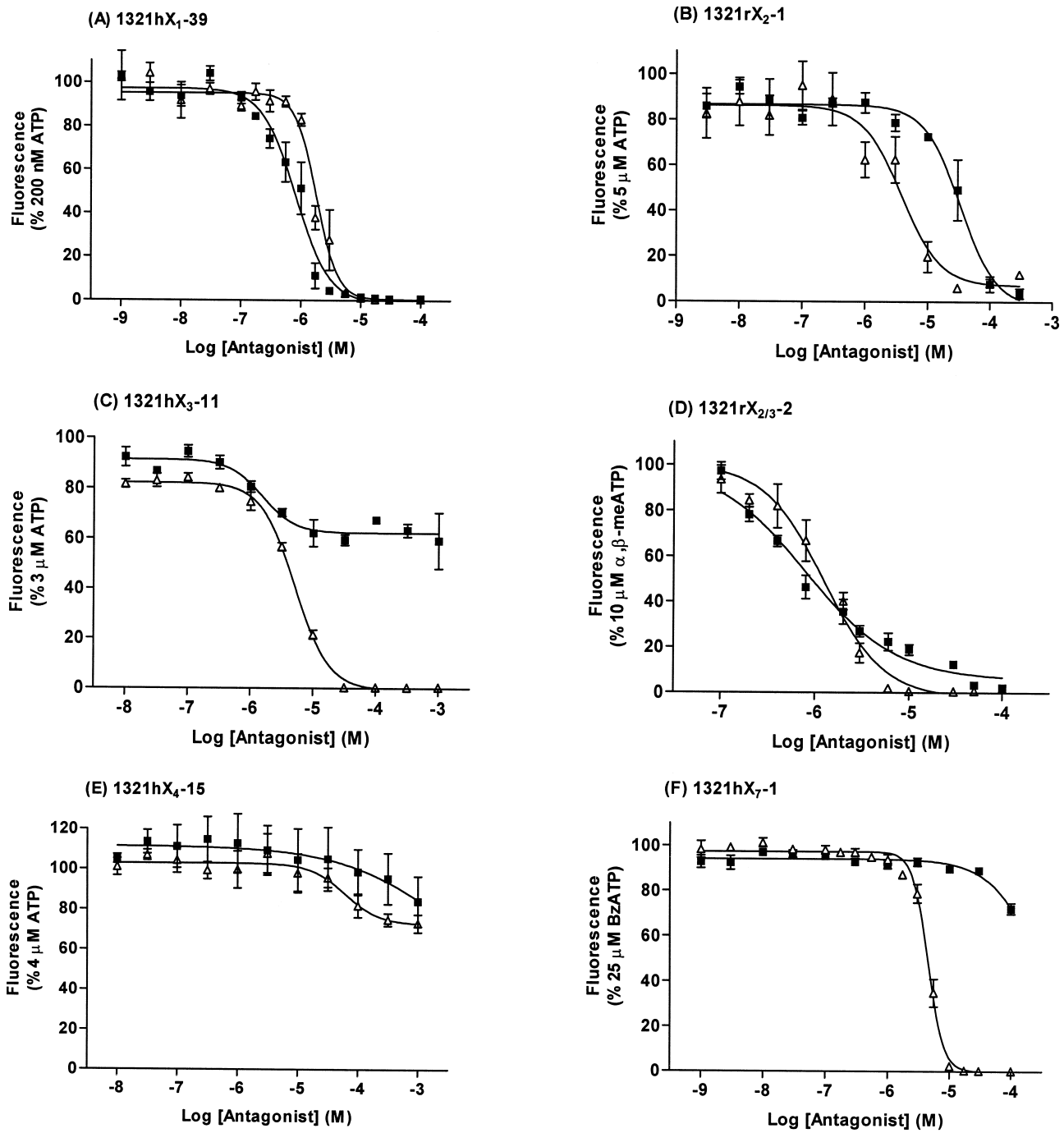

Fig. 4. Functional characterization of P2X receptor antagonists. P2X receptor-expressing cell lines were pre-incubated with suramin (■) or PPADS (△) for 3 min prior to activation with agonists as follows: (A) 1321hX$_1$-39 (200 nM ATP), (B) 1321rX$_2$-1 (5 μM ATP), (C) 1321hX$_3$-11 (3 μM ATP), (D) 1321rX$_{2/3}$-2 (10 μM αβ-meATP), (E) 1321hX$_4$-15 (4 μM ATP), (F) 1321hX$_7$-1 (25 μM BzATP). Data are shown as the mean percentage of the maximum agonist response detected in the absence of antagonist ± S.E.M. ($n = 3$).

receptor signal, whereas PPADS was a full antagonist (Fig. 4C, Table 1). In contrast, suramin was fully efficacious as a rP2X$_3$ receptor antagonist ($pIC_{50} = 6.11$).

Consistent with previous reports (Lewis et al., 1995), the heteromeric receptor consisting of rP2X$_2$ and rP2X$_3$ subunits (1321rX$_{2/3}$-2 cells) shared the pharmacological properties of P2X$_3$ with respect to activation by nucleotide agonists (Fig. 3D, Table 1). The rank order potency of agonists was comparable to the human and rat P2X$_3$ receptors, although the absolute potencies were slightly lower for the P2X$_{2/3}$ heteromer. αβ-meATP, which is selective for P2X$_3$ and P2X$_{2/3}$ receptor activation versus P2X$_2$ receptor activation, mediated a submaximal response when compared to ATP, suggesting the presence of homo-

Table 1
Functional potencies of P2X receptor agonists and antagonists
Potencies ($p\mathrm{EC}_{50}$ and $p\mathrm{IC}_{50}$) are average values ± S.E. of 3–5 determinations.
Values in parentheses indicate maximal effect as compared to a full ATP response.

$p\mathrm{EC}_{50}$	$\mathrm{P2X}_1$	$\mathrm{P2X}_2$	$\mathrm{P2X}_3$		$\mathrm{P2X}_{2/3}$	$\mathrm{P2X}_4$	$\mathrm{P2X}_7$[a]
	1321hX$_1$-39	1321rX$_2$-1	1321hX$_3$-11	1321rX$_3$-3	1321rX$_{2/3}$-2	1321hX$_4$-15	1321hX$_7$-1
Nucleotide agonists							
ATP	7.25 ± 0.05	5.85 ± 0.05	6.47 ± 0.04	6.47 ± 0.05	6.30 ± 0.04	6.32 ± 0.02	4.02 ± 0.01 (81%)
2-meS-ATP	7.27 ± 0.07	5.79 ± 0.05	6.45 ± 0.05	6.67 ± 0.06	6.00 ± 0.03	5.66 ± 0.07 (80%)	< 4
α,β-meATP	6.70 ± 0.07	< 4	6.13 ± 0.05	6.29 ± 0.05	5.47 ± 0.03 (71%)	5.08 ± 0.11 (50%)	< 4
ATPγS	5.64 ± 0.05 (80%)	5.89 ± 0.01	6.16 ± 0.04	6.15 ± 0.10	5.69 ± 0.02	4.96 ± 0.04 (61%)	< 4
BzATP	8.74 ± 0.10	5.26 ± 0.06 (90%)	7.10 ± 0.19 (90%)	7.50 ± 0.35	6.19 ± 0.10 (81%)	6.31 ± 0.05 (80%)	5.33 ± 0.02
ADP	4.98 ± 0.24	< 4	< 4	< 4	< 4	4.76 ± 0.24 (29%)	< 4
ADPβS	5.61 ± 0.04	< 4	5.06 ± 0.04	5.37 ± 0.10 (60%)	< 4	4.61 ± 0.04 (79%)	< 4
UTP	< 4	< 4	< 4	< 4	< 4	< 4	< 4
Diadenosine polyphosphates							
$p\mathrm{EC}_{50}$	$\mathrm{P2X}_1$	$\mathrm{P2X}_2$	$\mathrm{P2X}_3$		$\mathrm{P2X}_{2/3}$	$\mathrm{P2X}_4$	$\mathrm{P2X}_7$[a]
	1321hX$_1$-39	1321rX$_2$-1	1321hX$_3$-11	1321rX$_3$-3	1321rX$_{2/3}$-2	1321hX$_4$-15	1321hX$_7$-1
$\mathrm{AP}_2\mathrm{A}$	< 4	< 4	< 4	< 4	< 4	< 4	< 4
$\mathrm{AP}_3\mathrm{A}$	< 4	< 4	4.71 ± 0.10 (53%)	< 4	< 4	< 4	< 4
$\mathrm{AP}_4\mathrm{A}$	6.74 ± 0.12	< 4	6.30 ± 0.10 (64%)	6.10 ± 0.28 (34%)	4.32 ± 0.03	6.2 ± 0.07 (59%)	< 4
$\mathrm{AP}_5\mathrm{A}$	6.56 ± 0.03	< 4	6.21 ± 0.06 (81%)	5.23 ± 0.61	< 4	5.91 ± 0.07 (55%)	< 4
$\mathrm{AP}_6\mathrm{A}$	5.95 ± 0.08 (55%)	< 4	6.22 ± 0.06 (82%)	ND	< 4	4.20 ± 0.36 (60%)	< 4
Antagonists							
$p\mathrm{IC}_{50}$	$\mathrm{P2X}_1$	$\mathrm{P2X}_2$	$\mathrm{P2X}_3$		$\mathrm{P2X}_{2/3}$[b]	$\mathrm{P2X}_4$	$\mathrm{P2X}_7$[c]
	1321hX$_1$-39	1321rX$_2$-1	1321hX$_3$-11	1321rX$_3$-3	1321rX$_{2/3}$-2	1321hX$_4$-15	1321hX$_7$-1
Suramin	6.07 ± 0.05	4.48 ± 0.10	< 4	6.11 ± 0.26	6.09 ± 0.06	< 4	< 4
PPADS	5.74 ± 0.04	5.42 ± 0.13	5.29 ± 0.02	5.44 ± 0.08	5.90 ± 0.05	< 4	5.34 ± 0.01

[a] Maximal potency as compared to full BzATP response.
[b] Potency ($p\mathrm{IC}_{50}$) determined using 10 μM αβ-meATP as agonist.
[c] Potency ($p\mathrm{IC}_{50}$) determined using 25 μM BzATP as agonist.

meric $\mathrm{rP2X}_2$ receptors in 1321rX$_{2/3}$-2 cells. Ligands which exhibited agonist activity at both 1321rX$_2$-1 and 1321hX$_3$-11 cells (or cells expressing the $\mathrm{rP2X}_3$ receptor), such as ATP and 2-meS-ATP, also elicited maximal responses in 1321rX$_{2/3}$-2 cells (Table 1). In contrast to their activity at the $\mathrm{hP2X}_3$ receptor, diadenosine polyphosphates were significantly less potent as $\mathrm{rP2X}_{2/3}$ receptor agonists. Suramin and PPADS were active as full antagonists ($p\mathrm{IC}_{50} = 6.09$ and 5.09, respectively) (Fig. 4D, Table 1).

The $\mathrm{hP2X}_4$ receptor (1321hX$_4$-15 cells) was distinguished by its partial activation by purine analogs other than ATP ($p\mathrm{EC}_{50} = 6.32$) (Fig. 3E). BzATP was equipotent with ATP ($p\mathrm{EC}_{50} = 6.31$), but did not fully activate the $\mathrm{hP2X}_4$ receptor (80% of ATP). The putative P2X receptor-selective agonist, αβ-meATP, functioned as a relatively weak partial agonist ($p\mathrm{EC}_{50} = 5.08$), mediating a maximal response equal to 50% of ATP. $\mathrm{AP}_4\mathrm{A}$ was the most potent member of the family of diadenosine polyphosphates ($p\mathrm{EC}_{50} = 6.20$), but also functioned as a partial agonist (Table 1). Suramin and PPADS were inactive as $\mathrm{hP2X}_4$ receptor antagonists at concentrations up to 100 μM (Fig. 4E, Table 1).

The pore forming $\mathrm{hP2X}_7$ receptor mediates submaximal responses to ATP as compared to BzATP (Fig. 3F, Table 1), as previously shown (Gargett et al., 1997). All other nucleotide ligands, including the diadenosine polyphosphates, are functionally inactive as $\mathrm{hP2X}_7$ agonists (Table 1). Similar results were obtained with the rat $\mathrm{P2X}_7$ receptor (data not shown). The potencies of ATP and BzATP in mediating Ca^{2+} influx were similar to their ability to mediate membrane permeabilization, as measured by YO-PRO-1 influx (Fig. 3F). PPADS was active as a $\mathrm{hP2X}_7$ receptor antagonist ($p\mathrm{IC}_{50} = 5.36$), while suramin had no detectable effect on $\mathrm{hP2X}_7$ receptor activation (Fig. 4F, Table 1).

4. Discussion

P2X receptor subtypes were expressed and functionally characterized in a cellular background devoid of endogenous P2 receptor activity (1321N1 human astrocytoma cells). The kinetics of receptor activation, as measured by electrophysiological methods and by Ca^{2+} influx, are consistent with previously reported observations (see references in Section 1). $\mathrm{hP2X}_1$ and $\mathrm{hP2X}_3$ receptors exhibit rapid desensitization kinetics, whereas $\mathrm{rP2X}_2$, $\mathrm{rP2X}_{2/3}$,

$hP2X_4$ and $hP2X_7$ receptors desensitize slowly, if at all. Thus, the stable P2X receptor-expressing cell lines and Ca^{2+} influx methodology described here serve as a representative assay system for the pharmacological characterization of P2X receptor subtypes.

The recombinant human $P2X_1$ receptor expressed in 1321N1 cells exhibited kinetic and pharmacological properties similar in most respects to those previously described for the recombinant rat and human receptor subtypes (Valera et al., 1994; Evans et al., 1995). ATP, the endogenous ligand for P2X receptors, was at least five times more potent at $hP2X_1$ than at any of the other P2X receptors tested. Similarly, αβ-meATP, which has previously been described as a P2X-selective agonist (versus P2Y), was more potent at the $hP2X_1$ receptor than any other P2X receptor. However, BzATP, which has been widely reported as a $P2X_7$ receptor-selective agonist (Wiley et al., 1996), was a potent agonist at the $hP2X_1$ and rat and human $P2X_3$ receptors (Table 1). Thus, BzATP is primarily selective for $hP2X_1$ receptor activation, exhibiting at least 15-fold selectivity over any of the other P2X receptors tested and more than 2500-fold selectivity over the $hP2X_7$ receptor. Therefore, the use of BzATP as a $P2X_7$ receptor agonist in native tissues is complicated by its lack of selectivity and specificity for that receptor subtype. The present data indicate that most of the purine triphosphate P2 agonists described to date are more selective for the $P2X_1$ receptor.

Investigation of the $P2X_2$ receptor in vivo is complicated by the identification of up to six different splice variants of the rat homolog of the $P2X_2$ receptor (Simon et al., 1997; Koshimizu et al., 1998). Furthermore, the functional properties of the $rP2X_2$ clone described here were sensitive to modulation by extracellular pH and Zn^{2+} (data not shown), as has been previously described (King et al., 1996; King et al., 1997; Wildman et al., 1998). In the present studies, the characterization of the full-length $rP2X_2$ receptor ($P2X_{2a}$) was carried out at physiological pH (pH 7.4) and in the absence of added Zn^{2+}. $P2X_2$ receptor activation leads to a large and non-desensitizing transmembrane current and Ca^{2+} influx. ATP, 2-meS-ATP, ATPγS and BzATP function as equipotent agonists at the $hP2X_2$ receptor, whereas αβ-meATP is inactive (Table 1).

The $P2X_3$ receptor subtype is expressed primarily in sensory ganglia, and is, in some cases, co-localized with $P2X_2$ receptors in primary afferent neurons of the nodose, trigeminal and dorsal root ganglia (Chen et al., 1995; Lewis et al., 1995; Vulchanova et al., 1997). These two receptor subtypes are differentiated primarily on the basis of their desensitization kinetics and sensitivity to αβ-meATP (Lewis et al., 1995). In contrast with the $P2X_2$ receptor, $P2X_3$ receptor activation produced a rapidly desensitizing transmembrane current and Ca^{2+} influx which rapidly returns to baseline in the continued presence of agonist (Fig. 1CFig. 2C). Furthermore, like $P2X_1$ receptors, $P2X_3$ receptors are sensitive to activation by αβ-meATP. The rat and human homologs of the $P2X_3$ receptor exhibit similar pharmacological profiles for nucleotide agonists (Table 1).

Studies using whole cell recording techniques have shown that endogenous P2X receptors expressed in sensory neurons exhibit functional and pharmacological profiles unlike either the $P2X_2$ or the $P2X_3$ receptor subtypes (Khakh et al., 1995; Lewis et al., 1995). These receptors were sensitive to activation by αβ-meATP but did not desensitize in the continued presence of agonist. These functional properties were reproducible in a recombinant system only when the $P2X_2$ and $P2X_3$ receptor subunits were co-expressed in the same cell, suggesting the formation of functional heteromultimeric P2X receptors (Lewis et al., 1995; Radford et al., 1997). The $1321rX_{2/3}$-2 cells described here, which express both the rat $P2X_2$ and $P2X_3$ receptor subtypes, exhibit αβ-meATP sensitivity and slow desensitization kinetics. αβ-meATP mediates a sub-maximal response when compared to ATP in these cells. Since αβ-mATP selectively activates $rP2X_3$ and $rP2X_{2/3}$ receptors, the greater response to ATP relative to αβ-meATP is presumably due to the additional presence of homomeric ATP-sensitive $rP2X_2$ receptors in $1321rX_{2/3}$-2 cells (Fig. 1DFig. 2D).

Nucleotide agonists which activate the $P2X_1$, $P2X_2$ and $P2X_3$ homomeric receptors exhibit full efficacy with varying degrees of potency (Fig. 3). In contrast, the $P2X_4$ receptor is distinguished by its sub-maximal response to nucleotide analogs other than ATP (Fig. 3E, Table 1). Although $P2X_4$ receptor subunits have been shown to form heteromers with $P2X_6$ receptor subunits in vitro, the pharmacological and kinetic properties appear to remain unchanged (Le et al., 1998).

The $P2X_7$ receptor is functionally unique among the P2 receptors by virtue of its ability to form large transmembrane pores permeable to solutes up to 900 Da (Chiozzi et al., 1996; Chessell et al., 1997). Although the $P2X_7$ receptor shares sequence homology with other P2X receptors (Rassendren et al., 1997), it exhibits weak sensitivity to its putative physiological agonist ATP (Chessell et al., 1997). Our observation that ATP is a weak partial agonist in $1321hX_7$-1 cells is consistent with previous reports (Gargett et al., 1997). BzATP is 13-fold more potent than ATP in activating $hP2X_7$ receptor-mediated Ca^{2+} flux (Fig. 1), transmembrane current flow and membrane permeabilization (data not shown).

Diadenosine polyphosphates have been implicated in intercellular signaling in pancreas (Ripoll et al., 1996), adrenal gland (Pintor et al., 1992) and brain (Pintor and Miras-Portugal, 1995). At least three diadenosine polyphospates, AP_4A, AP_5A and AP_6A, have been suggested to function as neurotransmitters (Miras-Portugal et al., 1998). Diadenosine polyphosphates exhibit agonist activity at $P2X_2$ and $P2Y_1$ receptors (Pintor et al., 1996) and have been reported to mediate Ca^{2+} flux via receptors distinct from those for ATP (Pintor et al., 1997). The present data

indicate that AP_4A, AP_5A and AP_6A function as potent agonists at the $hP2X_1$, $hP2X_3$, and $hP2X_4$ receptor subtypes (Table 1). Diadenosine polyphosphates exhibit no detectable agonist activity at $rP2X_2$ and $hP2X_7$ receptors. AP_4A is a partial agonist at the $rP2X_3$ receptor while AP_5A exhibits full agonist activity. Interestingly, AP_5A is not active at the heteromeric $rP2X_{2/3}$ receptor, despite the observation that the $P2X_{2/3}$ and $P2X_3$ receptors are otherwise pharmacologically similar. Although these data do not rule out the existence of a receptor selective for diadenosine polyphosphates, they do suggest additional mechanisms by which these signaling molecules may mediate their physiological effects.

The paucity of selective P2 antagonists has complicated the identification and characterization of P2 receptor subtypes in native tissues. Suramin and PPADS have been routinely employed as putative P2-selective antagonists (Connolly, 1995; Bultmann et al., 1996), despite their lack of selectivity and specificity (Chen et al., 1996). The present data indicate that suramin and PPADS function as full antagonists only at the $hP2X_1$, $rP2X_2$, $rP2X_3$ and $rP2X_{2/3}$ receptors (Fig. 4, Table 1). Suramin exhibited weak partial inhibition of the $hP2X_3$ receptor (Fig. 4C) under the conditions described here. However, in the presence of sub-maximal concentrations of ATP, suramin functioned as a full antagonist (data not shown) in a manner consistent with previously reported data showing that suramin is a slowly-equilibrating and competitive P2X receptor antagonist (Leff et al., 1990). Neither suramin nor PPADS exhibited inhibitory effects at the $hP2X_4$ receptor. At the $hP2X_7$ receptor, PPADS functioned as a full antagonist, while suramin exhibited no detectable inhibitory activity.

The data presented here provide the first direct comparison of the kinetic and pharmacological properties of P2X receptors expressed in a uniform and silent background. Although P2 receptors share an endogenous agonist in vivo, they may function differentially on the basis of tissue distribution, activation kinetics, and quaternary structure (i.e., heteromultimerization). All of the prototypic P2X receptor agonists studied interacted non-specifically with multiple P2X receptor subtypes. Particularly striking was the observation that the putatively selective $P2X_7$ receptor agonist BzATP was 100- to 1000-fold more potent at the $P2X_3$ and $P2X_1$ receptors than the $P2X_7$ receptor. The observed activity of diadenosine polyphosphates as agonists at P2X receptors may provide a mechanism for some of their in vivo activities, although the existence of an additional AP_4A-selective receptor has been suggested (Pintor et al., 1997). These observations, as well as the inability of P2 receptor antagonists such as suramin and PPADS to differentiate between P2 receptor subtypes, emphasizes the need for selective and potent non-nucleotide ligands for use as pharmacological tools to further elucidate the functional significance of P2 receptor activation.

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