Cellular fluorescent high-throughput screening assays of the ATP-gated P2X₇ receptor

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The P2X₇ receptor (P2X₇R) is an important member of the P2X family of ligand-gated ion channels that respond to ATP as the endogenous agonist. Studies suggest that P2X₇R plays a potentially pivotal role in a variety of physiological functions, including peripheral and central neuronal transmission, smooth muscle contraction, and inflammation. Thus, P2X₇R may be a potential target for drug development. Here, we used a FlexStation to examine the function of recombinant P2X₇R stably expressed in human embryonic kidney 293 cells and to compare three high-throughput screening assays: a membrane potential assay, an ethidium bromide uptake assay, and a calcium influx assay. We found that all three assays were suitable for the analysis of P2X₇R, but the calcium influx assay was the most robust and is the best choice as a first high-throughput screening assay when embarking on a P2X₇R drug discovery project.

P2X₇R, high-throughput screening, FlexStation, calcium influx assay, membrane potential assay, ethidium bromide (EtBr) uptake assay

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The P2X₇ receptor (P2X₇R) belongs to the P2X family of ligand-gated ion channels. These channels respond to ATP (adenosine-5'-triphosphate) as the endogenous ligand and differ in their distribution, gating properties, and affinity for ATP and ATP analogs [1,2]. P2X₇Rs are primarily expressed in epithelial cells and antigen-presenting cells such as lymphocytes, mast cells, and macrophages, where they exert immunomodulatory functions including inflammatory cytokine release, membrane fusion events, and apoptosis [3–5]. Most recently, the manufacture of several P2X₇R antagonists with improved “drug-like” properties was reported; for instance, AZD9056 has already entered Phase II clinical trials for rheumatoid arthritis [6–8]. A-740003 exhibited preclinical efficacy as a P2X₇R antagonist in rodent models of neuropathic pain [9]. These studies have suggested that the P2X₇R signaling pathway could be a therapeutic target for treating various neurodegenerative diseases,

rheumatoid arthritis, and neuropathic pain [10–12].

Until now, there have been very limited options to measure the pharmacological properties of P2X₇Rs. P2X₇Rs respond to brief (≤ 1 s) stimulation with extracellular agonist by the fast opening of an intrinsic cation-conducting pore, permitting the selective permeation of small cations (Na⁺, K⁺, and Ca²⁺) into the cell [13]. As a result, the plasma membrane depolarizes and the Na⁺ and K⁺ gradients collapse. Both the actual ion influx and the change in membrane potential can be exploited for the development of functional assays for P2X₇R screening.

The gold-standard technology to study ion channels is electrophysiology using whole-cell voltage-clamp recordings. It is sensitive and reliable, and offers the most accurate and high-content data. However, depending on many factors such as the cell type and the type of ion channel under study, patch clamping can be very labor-intensive and has a very low throughput.

Fluorescent dyes sensitive to changes in membrane po-

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tential have been improved significantly during the last few years. The FLIPR system from Molecular Devices (Sunnyvale, CA, USA) can measure changes in membrane potential in high-throughput screening (HTS) format. The FLIPR system uses a 530-nm argon laser for excitation of the fluorescent dye that exhibits an increase in the emission intensity at 565 nm when the membrane depolarizes.

Ion-specific fluorescent probes, such as calcium indicator dyes, are also available for measuring the ionic concentrations in cells. A no-wash calcium assay kit from HD Biosciences (Shanghai, China) can measure the fluorescent signal of calcium with a FlexStation system (Molecular Devices) or the FLIPR system in HTS format.

A particular feature of P2X₇Rs is that prolonged or repeated activation promotes the formation of large pores; these pores allow the bi-directional passage of molecules into the cell, as indicated by the entry of organic cations of up to 900 Da, and the leakage of metabolites [14,15]. Ethidium bromide (EtBr), a fluorescent, DNA-binding dye with a molecular weight of 314 Da, could enter through the dilated or "large-pore" form of the P2X₇R and bind to intracellular DNA/RNA, whereupon it increases its fluorescence intensity many fold. While this general method has been used to study P2X₇R function in suspensions of cells in fluorimeter cuvettes, we have found that it is possible to perform this assay using cells in a 96-well plate, thus providing a relatively simple and robust functional assay for P2X₇R screening in HTS format.

Here we present data obtained from characterizing recombinant P2X₇R expressed in human embryonic kidney (HEK) 293 cells. To select the best HTS assay to identify P2X₇R ligands, we developed three HTS assays: a membrane potential assay, an EtBr uptake assay, and a calcium influx assay. For pharmacological comparisons, we investigated known P2X₇R agonists in these three assays and compared the data to those from patch-clamp assays. Our results showed that all three HTS assays were suitable for the identification of P2X₇R ligands, but the calcium influx assay was the most robust and is the most suitable for a first HTS assay when embarking on a P2X₇R drug discovery project.

1 Materials and methods

1.1 Materials

All cell culture media and supplements were obtained from Gibco (Invitrogen, Carlsbad, CA, USA), except fetal bovine serum, which was from Hyclone (Erembodegem-Aalst, Belgium). Blue membrane potential dye was purchased from Molecular Devices. The no-wash calcium assay kit was purchased from HD Biosciences. ATP, Bz-ATP (3'-O-(4-benzoyl)-benzoyl adenosine-5'-triphosphate) and the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2 Generation of a cell line stably expressing human P2X₇Ri

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (with 4.5 mg/mL D-glucose) containing 2 mmol/L glutamine and 10% fetal bovine serum. The cDNA encoding human P2X₇R was synthesized based on the published sequence (NCBI accession No. NM_002562) and inserted into the expression vector pCDNA3.1-hygro(+). Transfection of HEK 293 cells was performed using Lipofectamine™ (Invitrogen) according to the manufacturer's instructions. Cells were incubated with the DNA-plus-Lipofectamine complex at 37°C for 24 h in a humidified 5% CO₂ atmosphere. The cells were then passaged into fresh culture medium and selected with 100 µg/mL hygromycin. Stable clones were grown in the same medium and were chosen based on their functional responses to Bz-ATP (10 µmol/L) in a Ca²⁺ influx assay. The cells were maintained in a 37°C incubator in a humidified 5% CO₂ atmosphere.

1.3 RT-PCR analysis

Total RNA was isolated from HEK/hP2X₇R cells using the RNA isolation reagent Trizol (Invitrogen) according to the manufacturer's recommendations. For cDNA synthesis, 2 µg of total RNA was used in the RT reaction with 1 µg of oligo (dT) primer, heated for 5 min at 70°C, and then 5× PCR buffer, 0.5 mmol/L dNTP (dATP, dGTP, dCTP, and dTTP) mix, and 1 unit of RNase inhibitor were added and incubated at 42°C for 5 min. The 200 U of SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA) were added, and the tube was incubated at 37°C for 60 min. The reaction was then heated to 70°C to inactivate the reverse transcriptase. To amplify a 460-bp fragment of P2X₇R, we used the forward primer 5'-TCACTGCCGTC-CCAAATACAGTTTCCG-3' (nucleotide position 801–827) and the reverse primer 5'-TGCTGGTTCACCATCCTA-ATGTGGG-3' (nucleotide position 1220–1244).

1.4 Electrophysiology

Whole cell recordings were made using an EPC10 patch-clamp amplifier and pulse acquisition programs (HEKA, Lambrecht, Germany). Patch pipettes (5–7 MΩ) contained 154 mmol/L CsCl, 10 mmol/L EGTA, and 5 mmol/L HEPES. The "low divalent" extracellular solution contained 147 mmol/L NaCl, 2 mmol/L KCl, 0.3 mmol/L CaCl₂, 10 mmol/L HEPES, and 12 mmol/L glucose. Agonists were delivered by a voltage command 8 pinch valve superfusion system (ALA Scientific Instruments, Farmingdale, NY, USA). Experiments were carried out at room temperature.

1.5 Determination of membrane potential changes

HEK 293 cells stably expressing hP2X₇R were plated into

poly-*D*-lysine-coated 96-well clear-bottom black-walled plates (BD Biosciences, Bedford, MA, USA) at 40000 cells/well in 100 μ L of growth medium and grown for 24 h prior to testing. Cells were washed with Hank's balanced salt solution (Invitrogen) with 1 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$, leaving 50 μ L of buffer per well after the last wash. Blue membrane potential dye (50 μ L) was added, and the cells were incubated with dye for 45 min at 37°C. After incubation, the dye-loaded cell plate was placed in the FlexStation and the whole plate was imaged every 2 s (excitation at 488 nm and emission at 550 nm); then after 20 s, agonist was added to the cells. To determine the effect of P2X₇R blockers, cells were pretreated with compounds for 5–10 min before the plate was transferred to the FlexStation.

1.6 EtBr uptake analysis

EtBr uptake was carried out as previously described [14] with some modifications. Briefly, HEK 293 cells stably expressing human P2X₇R were harvested and seeded into poly-*D*-lysine-coated 96-well clear-bottom black-walled plates (BD Biosciences) at 20000 cells/well in 100 μ L culture medium, 24 h prior to the assay. Before the assay, the culture medium was removed and the cells were washed twice with dye uptake buffer (280 mmol/L sucrose, 5.6 mmol/L KCl, 0.5 mmol/L $CaCl_2$, 10 mmol/L glucose, 10 mmol/L HEPES, and 5 mmol/L *N*-methyl-*D*-glucamine, pH 7.4), then the cells were incubated in 100 μ L of dye uptake buffer containing 100 μ mol/L EtBr. Cells were subsequently treated with different concentrations of Bz-ATP (100 μ L/well) prepared in dye uptake-EtBr buffer. After a 30-min incubation, the EtBr uptake was monitored using a fluorometric imaging plate reader FlexStation with an excitation wavelength of 525 nm and an emission wavelength of 610 nm. Cells were pretreated with various concentrations of antagonists for 15 min at 37°C before the addition of Bz-ATP (10 μ mol/L).

1.7 Determination of Ca^{2+} influx

HEK 293 cells stably expressing hP2X₇R were plated into poly-*D*-lysine-coated 96-well clear-bottom black-walled plates (BD Biosciences) at 40000 cells/well in 100 μ L growth medium and grown for 24 h prior to testing. The medium was removed and the cells were incubated for 1 h at 37°C in 100 μ L loading buffer (280 mmol/L sucrose, 5.6 mmol/L KCl, 0.5 mmol/L $CaCl_2$, 10 mmol/L glucose, 10 mmol/L HEPES, and 5 mmol/L *N*-methyl-*D*-glucamine, pH 7.4, and 1 mmol/L probenecid containing 1 $\times$ calcium no-wash dye (BD Biosciences)). After incubation, the dye-loaded cell plate was placed in the FlexStation, and the whole plate was imaged every 2 s (excitation at 485 nm and emission at 525 nm); then after 20 s, 25 μ L of 5 $\times$ agonist was added to the cells. To determine the effect of the P2X₇R blockers, the cells were pretreated with compounds for 15

min before the plate was transferred to the FlexStation. The fluorescent signal was quantitated in relative fluorescence units (RFU) as $RFU_{max} - RFU_{min}$ over the 2-min run time.

1.8 Data analysis

Fluorescence signals in the Ca^{2+} influx assay were acquired for 90 s and then analyzed using SoftMax Pro software (version 4.8; Molecular Devices). The initial 10 s of the baseline were used to zero the signal, and the remaining signal was integrated using the area under the curve function.

The EC₅₀ and IC₅₀ values were determined from eight-point concentration–response curves. All curve fittings were performed using GraphPad Prism version 4 software (GraphPad Software Inc., San Diego, CA, USA). The Z factor was calculated according to the method developed by [16]

$$Z = 1 - 3 \times (|SD_{positive}| + |SD_{negative}|) / (|Mean_{positive}| - |Mean_{negative}|),$$

where $SD_{positive}$ and $SD_{negative}$ are the standard deviations of the positive and negative controls, respectively, and $Mean_{positive}$ and $Mean_{negative}$ are the means of the positive and negative controls, respectively.

2 Results and discussion

2.1 Molecular analysis of the hP2X₇R transcript

While generating the stable HEK/hP2X₇R cell lines, we first looked for the presence of mRNA expression of hP2X₇R. The hP2X₇R RT-PCR products migrated at 460 bp (Figure 1). As predicted, after treatment with *Bam*H I, the 460-bp PCR product was digested to 150- and 310-bp fragments.

2.2 Electrophysiological properties of hP2X₇R in HEK 293 cells

The expression of hP2X₇R was also confirmed by whole-cell electrophysiological recordings. Brief application (1–3 s)

Figure 1 Agarose gel electrophoresis (2% agarose) of RT-PCR products. A single 460-bp hP2X₇R product was observed at the predicted position. 1: cDNA from wild-type HEK 293 cells; 2: cDNA from a HEK/hP2X₇R stable cell line; 3: digestion of the 460-bp PCR product with *Bam*H I.

of Bz-ATP to HEK 293 cells transfected with hP2X₇R evoked inward currents (at -70 mV) and the EC₅₀ value of Bz-ATP was 110 $\mu\text{mol/L}$ (Figure 2). In non-transfected HEK 293 cells, application of a high concentration of Bz-ATP produced no significant inward currents (< 50 pA; data not shown). These results indicated that the transfected cell line expresses functional hP2X₇R.

2.3 Functional characterization of hP2X₇R using a membrane potential assay

The increase in Na⁺ influx associated with activation of hP2X₇R causes a concomitant transient change in membrane potential, which can be measured using fluorescent dyes sensitive to changes in membrane potential. The feasibility of a membrane potential assay to identify hP2X₇R channel function was explored using Molecular Devices' blue membrane potential-sensitive dye detected with a FlexStation. Activation of hP2X₇R channels with Bz-ATP resulted in an increase in fluorescent signal using membrane potential dye. Typical responses are shown in Figure 3. Both Bz-ATP and ATP caused a dose-dependent change in the fluorescent signal due to a hyperpolarization of the cellular membrane with EC₅₀ values of 27.3 $\mu\text{mol/L}$ and 169.2 $\mu\text{mol/L}$, respectively (Figure 4). The EC₅₀ for Bz-ATP was lower than the values obtained using electrophysiological data; however, the agonists' (ATP and Bz-ATP) rank order of potency was compatible with reference data [8].

2.4 Functional characterization of hP2X₇R using an EtBr uptake assay

Addition of agonist to cell suspensions of HEK 293 cells expressing hP2X₇R caused a time- and concentration-dependent increase in EtBr fluorescence (Figure 5). This response was not observed in non-transfected HEK 293 cells even when the cells were incubated in EtBr assay buffer for 1 h and stimulated with 50 $\mu\text{mol/L}$ ATP.

Next, we analyzed the dose responses of the three agonists, Bz-ATP, ATP, and 2-MesATP. Both the efficacy of Bz-ATP, ATP, and 2-MesATP, and their rank order of potency were compatible with reference data [14]. Among these agonists, Bz-ATP was the most potent, with an EC₅₀ of 4.22 $\mu\text{mol/L}$; ATP was less potent, with an EC₅₀ of 42.88 $\mu\text{mol/L}$; and 2-MesATP was the least potent, with an EC₅₀ of 85 $\mu\text{mol/L}$ (Figure 6).

We also analyzed the dose responses of the specific hP2X₇R blocker KN62 (1-[N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine). The potency of KN62 was compatible with reference data [14], with an IC₅₀ value of 437 nmol/L (Figure 6(d)).

2.5 Functional characterization of hP2X₇R using a Ca²⁺ influx assay

As a ion channel receptor, P2X₇R can respond to extracellular agonists by the fast opening of an intrinsic cation-conducting pore, permitting the selective permeation of

Figure 2 Bz-ATP-activated currents in HEK 293 cells expressing hP2X₇R. (a) Recording from one cell in response to application of nearly maximal concentrations of Bz-ATP in a solution containing 0.3 mmol/L CaCl₂ and no magnesium (low divalents). (b) Concentration-response curves for Bz-ATP-induced currents in low divalent external solution. $n=5$ for each point. (c) Superimposed current traces obtained from one cell in response to the application of different concentrations of Bz-ATP.

Figure 3 Typical hP2X₇R FlexStation response in HEK 293 cells loaded with membrane potential dye. (○) Untransfected HEK 293 cells; (□) HEK 293 cells stably expressing hP2X₇R. Bz-ATP (100 μmol/L) was added at 16 s.

Figure 4 Dose responses of compounds in the hP2X₇R-expressing cell line. Assays were performed in duplicate and monitored with a FlexStation plate reader. See the Materials and Methods for details of the assay. (a) Bz-ATP; (b) ATP. The data points represent means ± SEM. EC₅₀ values were determined using GraphPad Prism 4 software.

Figure 5 Effect of agonist-stimulated increases in EtBr fluorescence in HEK/hP2X₇R cells. (a) Fluorescent view of cells; (b) normal view of HEK/hP2X₇R cells. 1: HEK/hP2X₇R cells stimulated with 10 μmol/L 2-MesATP (2-methylthioadenosine triphosphate tetrasodium); 2: HEK/hP2X₇R cells stimulated with 10 μmol/L Bz-ATP; 3: HEK/hP2X₇R cells stimulated with 50 μmol/L ATP; 4: dye uptake buffer only; 5: wild-type HEK 293 cells stimulated with 50 μmol/L ATP. See the Materials and methods for details of the assay.

small cations (Na⁺, K⁺, and Ca²⁺) into the cell. A no-wash calcium assay kit was used to measure the fluorescent signal of calcium with a FlexStation system.

Activation of hP2X₇R channels with Bz-ATP resulted in a seven- to nine-fold increase in fluorescent signal using a calcium indicator dye. Typical responses are shown in Figure 7. We also analyzed the dose responses of the three agonists,

Bz-ATP, ATP, and 2-MesATP. The potency of each was compatible with reference data [8]. Both the efficacy of Bz-ATP, ATP, and 2-MesATP, and their rank order of potency were consistent with published data [8] and the results of the EtBr uptake assay. Among the agonists we tested, Bz-ATP was the most potent, with an EC₅₀ of 10.54 μmol/L; ATP was less potent, with an EC₅₀ of 50.8 μmol/L; and

Figure 6 Dose responses in an hP2X₇R-expressing cell line. Assays were performed in duplicate and monitored with a FlexStation plate reader. See the Materials and methods for details of the EtBr uptake assay. (a) Bz-ATP; (b) ATP; (c) 2-MesATP; (d) KN62. The data points represent the means $\pm$ SEM. The EC₅₀ or IC₅₀ values were determined using GraphPad Prism 4 software.

Figure 7 Typical hP2X₇R FlexStation responses in HEK 293 cells loaded with calcium indicator dye. (□) Untransfected HEK 293 cells; (○) HEK 293 cells stably expressing hP2X₇R. Bz-ATP (100 μ mol/L) was added at 16 s.

2-MesATP was the least potent, with an EC₅₀ of less than 55 μ mol/L (Figure 8). We also performed dose responses for the specific blocker hP2X₇R KN62 using this Ca²⁺ influx assay. The potency of KN62 was compatible with reference data [9]; with an IC₅₀ value of approximately 437 nmol/L (Figure 8(d)).

2.6 Evaluation of the Ca²⁺ influx assay in HTS format

We found that all three fluorescent assays were suitable for analyzing hP2X₇R, but the calcium influx assay was the most robust, considering the weak signals in the membrane potential assay and the extra dye-wash step required in the

EtBr dye uptake assay.

To show the robustness of the Ca²⁺ assay, the fluorescence signals for individual wells in a 96-well plate were plotted (Figure 9(b)). The average signals were $42.46 \times 10^5 \pm 2.27 \times 10^5$ RFU for Bz-ATP and $4.29 \times 10^5 \pm 0.32 \times 10^5$ RFU for buffer ($n=48$). The calculated signal-to-background ratio was 19.36, and the Z factor was 0.796.

The Z factor was also calculated in 10 consecutive 96-well plates to assess inter-plate variation. Table 1 shows the Z factor for a 1-day run with 10 plates. The Z factors were very consistent between plates throughout the day.

In summary, we investigated different fluorescent assay formats for studying hP2X₇R: a membrane potential assay,

Figure 8 Dose responses of agonists in an hP2X₇R-expressing cell line. Assays were performed in duplicate and monitored with a FlexStation plate reader. See Materials and Methods for details of the calcium influx assay. (a) Bz-ATP; (b) ATP; (c) 2-MesATP; (d) KN62. The data points represent the means ± SEM. The EC₅₀ or IC₅₀ values were determined using GraphPad Prism 4 software.

Figure 9 Z factor determination in 96-well plate format. A HEK/hP2X₇R cell line was stimulated with 30 μmol/L Bz-ATP (EC₁₀₀ as signal) and vehicle (0.2% dimethyl sulfoxide as background). (a) Raw data for the Z factor test; (b) Scatter plot of the data. See Materials and methods for details of the calcium influx assay. The calcium signal detection time for each column was 80 s.

Table 1 Individual plate Z factors in 10 consecutive 96-well plates ^{a)}

Plate No.	1	2	3	4	5	6	7	8	9	10
Z factor	0.834	0.898	0.874	0.861	0.908	0.882	0.831	0.866	0.793	0.870

a) HEK/hP2X₇R cells were plated in 10 consecutive 96-well plates. Vehicle (0.2% dimethyl sulfoxide) and 30 μmol/L Bz-ATP (EC₁₀₀) were applied to obtain the background and the signal readings, respectively. The Z factor was calculated for each individual plate. See Materials and methods for details of the calcium influx assay. The calcium signal detection time for each column was 80 s.

a dye uptake assay and a Ca²⁺ influx assay. Similar pharmacological properties were observed for hP2X₇R-expressing HEK 293 cells in these three fluorescent assays. However, the Ca²⁺ influx assay gave a much bigger assay window than the membrane potential assay, and required fewer steps than the EtBr uptake assay. The Ca²⁺ influx assay performance was very consistent and there was little variation in Z factor and Signal/Background from plate to plate and from day to day. The Ca²⁺ influx assay enabled us to embark on a P2X₇R drug discovery project.

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