Human neutrophils do not express purinergic P2X₇ receptors

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Abstract It has been reported that in human neutrophils, external ATP activates plasma membrane purinergic P2X₇ receptors (P2X₇R) to elicit Ca²⁺ entry, production of reactive oxygen species (ROS), processing and release of pro-inflammatory cytokines, shedding of adhesion molecules and uptake of large molecules. However, the expression of P2X₇R at the plasma membrane of neutrophils has also been questioned since these putative responses are not always reproduced. In this work, we used electrophysiological recordings to measure functional responses associated with the activation of membrane receptors, spectrofluorometric measurements of ROS production and ethidium bromide uptake to assess coupling of P2X₇R activation to downstream effectors, immune-labelling of P2X₇R using a fluorescein isothiocyanate-conjugated antibody to detect the receptors at the plasma membrane, RT-PCR to determine mRNA expression of P2X₇R and Western blot to determine protein expression in neutrophils and HL-60 cells. None of these assays reported the presence of P2X₇R in the plasma membrane of neutrophils and non-differentiated or differentiated HL-60 cells—a model cell for human neutrophils. We concluded that P2X₇R are not present at plasma membrane of human neutrophils and that the putative physiological responses triggered by external ATP should be reconsidered.

Keywords Human · Neutrophils · Purinergic receptors · Protein · Whole cell current

Introduction

The first line of defence in the human immune system comprised neutrophils, basophils and eosinophils. Neutrophils are able to recognise and phagocytise invading microorganisms, bacterial components and opsonised particles [3, 29]. Neutrophils carry out these functions by adhering to endothelial cells, migrating through tissues to reach the site of infection and then producing reactive oxygen species and releasing content of their granules to destroy the invading microorganisms [3].

In blood, the ATP released by healthy cells in response to stimuli such as shear stress and cell swelling, by damaged cells, by vascular injury or by stimulated platelets, [14, 34] is able to stimulate several leukocyte functions such as DNA synthesis, blastogenesis, cell-mediated killing, apoptosis, pro-inflammatory activities, adhesion to endothelial cells, shedding of CD62L and expression of Mac1 [2, 28, 37, 40]. These effects are mediated by purinergic receptors that belong to the metabotropic (P2Y) and/or ionotropic (P2X₁–P2X₇) type [6, 25, 33]. In neutrophils, extracellular ATP modulates adhesion to endothelial cells by inducing the expression of CD11a and CD11b, as well as phagocytosis and apoptosis [5, 17, 30, 35, 41]. ATP can either inhibit cell motility [15] or stimulate it via P2Y₂ receptor activation [24, 45]. Finally, ATP together with the granulocyte macrophage colony-stimulating factor protects neutrophils from apoptosis, thus extending their life span [18].

It has been suggested that in neutrophils and HL-60 cells, the effects of external ATP on the production of reactive oxygen species (ROS) such as O₂^{•−} and H₂O₂ are

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mediated by the activation of P2X₇ receptors (P2X₇R) [42]. However, the expression of P2X₇R in the plasma membrane of neutrophils remains controversial. Although mRNA for P2X₇ as well as P2X₁ and P2X₄ has been detected by some groups in human neutrophils [8, 42], others failed to detect both mRNA and protein in whole human neutrophils [31, 44]. Furthermore, it has been suggested that most of P2X₇R are in the cytosol of human neutrophils serving as a reserve to be recruited to the plasma membrane after cell activation [19]. However, treatment of neutrophils with lipopolysaccharide (LPS), which is known to up-regulate P2X₇R expression in monocytes [22], did not induce P2X₇R protein expression [44]. Inconclusive results were also obtained with HL-60 cells differentiated with dimethylsulfoxide (DMSO), a cell line widely used as a neutrophil model. In these cells, both up-regulation of P2X₇R [42] and down-regulation of P2X₇R mRNA levels have been reported [11, 12].

Efforts to establish the functional role of P2X₇ receptors in neutrophils are complicated by the expression of at least 250 polymorphic forms of the receptor and by the lack of specific activators [13, 16]. Nevertheless, establishing the presence of P2X₇R in the plasma membrane beyond reasonable doubts is the first step towards understanding the physiological role of this purinergic receptor in neutrophils. In this paper, we evaluated the functional expression of P2X₇R in human neutrophils and HL-60 cells using techniques that measure directly the activity and expression of the receptor at the plasma membrane. Our data show that P2X₇R are not present in human neutrophils.

Materials and methods

Materials

ATP (di-Tris salt), Benzoyl-benzoyl ATP (BzATP), bovine serum albumin (BSA), phorbol myristate acetate (PMA), DMSO, apyrase and salts used to prepare saline solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH₂-DA) was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA) and 2',7'-dichlorofluorescein (DCF) from Fluka (Sigma). Reverse transcription polymerase chain reaction (RT-PCR) reagents, SuperScript II reverse transcriptase, oligo(dT)₁₂₋₁₈ primers, RNase OUT and Taq DNA Polymerase were purchased from Invitrogen. P2X₇R antibodies (APR-008, APR-008-F and APR-004) for Western blotting and immunostaining were from Alomone Labs (Jerusalem, Israel). The horseradish peroxidase (HRP)-conjugated secondary and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and cell transfection

Human leukemic promyelocytes HL-60 cells were grown in RPMI 1640 medium containing 10% foetal bovine serum (FBS; HyClone, Logan, Utah, USA) and 1% gentamicin (Gibco, Grand Island, NY, USA). HL-60 cells (10⁵ cells/ml) were treated with 1.3% (v/v) DMSO for 6 days to differentiate them into neutrophil-like cells [10]. Morphological changes were verified by Wright-giemsa staining. Mouse macrophage J774 cells were cultured in advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 0.5% fungizone, 200 mM L-glutamine and 1% penicillin–streptomycin. HEK-293 cells were cultured in DMEM containing 10% FBS, 1% gentamicin and 0.11 mg/5 ml sodium pyruvate (all from Gibco). Cells were detached from culture flasks using 0.1% trypsin in Hanks's balanced salt solution (HBSS, from Gibco). Cultures were kept at 37°C in a humid atmosphere with 5% CO₂. Cell count was maintained between 0.05 and 1 × 10⁶ cells/ml. HEK-293 cells were transfected with mouse P2X₇ (mP2X₇) cDNA using the lipofectin reagent according to instructions from the manufacturer [38].

Isolation of human peripheral blood neutrophils

Human neutrophils were isolated as previously described [23] from blood samples obtained from consenting healthy volunteers following an approved protocol (Bioethical and Research Committee/CBR 2003-001). Briefly, 2.5 ml of whole blood was collected in tubes containing sodium heparin (86 USP units) and layered over 2.5 ml of 1-Step Polymorphs reagent (Accurate Chemical & Scientific Co., Westbury, NY, USA). After one centrifugation at 500×g for 35 min, the layer containing the neutrophils was separated and washed at 200 g for 10 min in HBSS without Ca²⁺ and Mg²⁺ supplemented with 0.1% IgG-free BSA. Contaminating erythrocytes were eliminated by lysis with a hypotonic solution that contained either 0 or 2 U/ml of apyrase supplemented with 5 mM Ca²⁺. After the lysis step and before use, the neutrophils were washed twice in HBSS 0.1% BSA and centrifuged at 200×g during 10 min. Cell viability was determined by trypan blue staining; above 95% of the cells were viable in our preparation.

Patch-clamp recordings

P2X₇R is permeable to cations such as Na⁺, K⁺, Ca²⁺, NMDG⁺ and TEA⁺ as well as other ions such Cl[−] [33, 38]. In this work, we used solutions containing various cations to measure the currents activated by ATP or BzATP. Whole cell currents were recorded at room temperature (20–22°C) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Currents were filtered at 2 or 5 kHz

using the built-in Bessel filter and sampled using a Digidata 1200 analogue-to-digital board (Molecular Devices) installed in a personal computer. The pCLAMP package V8.0 (Molecular Devices) was used to deliver voltage commands, data acquisition and for analysis. Pipettes (Corning 8161, Warner Instruments Inc.; Hamden, CT, USA) had resistances between 3 and 5 M Ω when filled with the pipette (internal) solution containing (in mM): NaCl (or TEACl) 140, EGTA 20, HEPES 20 (pH 7.3; tonicity 335 mOsm/kg). Neutrophils and J774 cells were bathed in a standard external solution containing (in mM): NaCl (or TEACl) 140, CaCl₂ 0.5, D-mannitol 100, HEPES 20 (pH 7.3; tonicity 380 mOsm/kg). In those experiments carried out in the presence of high [Ca²⁺]_e, the external solution contained (in mM): NaCl 110, CaCl₂ 20, D-mannitol 100, HEPES 20 (pH 7.3; tonicity 380 mOsm/kg). Solutions containing Tris-ATP or BzATP were daily prepared and pH was readjusted to 7.3 with NaOH or TEAOH after adding any amount needed to reach the desired final concentration. Undifferentiated HL-60 cells were patched using pipettes filled with an intracellular solution containing (in mM) KCl 115, TEA-Cl 5, EGTA 10, HEPES 10 whose pH was adjusted to 7.3 with KOH and bathed with an external solution containing (in mM) TEACl 140, KCl 5 and HEPES 10 whose pH was adjusted to 7.3 with TEAOH. Solutions were gravity-perfused into the recording chamber at a flow rate of about 4 ml/min. Neutrophils, HL-60 cells and J774 cells were held at 0 mV and stepped to -80 mV during agonist application.

Ethidium uptake

Human neutrophils, HL-60 cells, differentiated HL-60 and P2X₇-transfected HEK-293 cells were plated onto 4-mm coverslips and bathed in a solution containing (in mM): TEACl 140, D-mannitol 100, HEPES 20, ethidium bromide 0.0006 (pH 7.3 with TEAOH). The coverslips were placed on the stage of a Nikon Eclipse TE2000 inverted microscope. Cells were observed with a Pan Fluor $\times 60$ objective and were excited with green light (528–553 nm) using a Nikon G-2E/C filter set and Nikon Super High Pressure Mercury Lamp. Images were digitised by a Hamamatsu C4742-95 camera attached to the side port of the microscope and analysed using the Imaging Workbench 6.0 software (Indec BioSystems, Santa Clara, CA, USA). Cells were incubated for at least 5 min in the solution containing ethidium in the absence of ATP to make sure that no ethidium uptake took place under basal conditions; otherwise, cells were excluded. After this period, cells were exposed to 5 mM ATP (bis-Tris salt) to maximally activate all purinergic receptors [7]. Background signal (average of at least three similar traces from cell-free regions located near the cell of interest) was subtracted during off-line analysis.

Spectrofluorometric measurement of ROS

ROS production was measured using DCFH₂-DA, a compound extensively used for this purpose in many cell types including neutrophils [4, 32, 46]. Neutrophils and differentiated HL-60 cells (10⁶ cells/ml) suspended in HBSS were loaded for 15 min with 5 μ M DCFH₂-DA in a water bath at 37°C with agitation. Unloaded dye was removed by centrifugation at 2,000 $\times g$ for 1 min. Then, the pellet was resuspended in HBSS without or with 2.2 mM CaCl₂ and incubated at 37°C for 5 min. Cells were stimulated with vehicle (control) or BzATP (100 μ M) or PMA (10 μ M) during 10 min at 37°C and then placed into a quartz cuvette for fluorescence measurement using a spectrofluorometer and FelixTM software version 1.42 (Photon Technology International, Lawrenceville, NJ, USA). Excitation and emission wavelengths were 501 and 522 nm, respectively. ROS oxidises DCFH to DCF which is a highly fluorescent molecule [27]. Thus, a calibration curve was constructed from fluorescence measurements of serial dilutions of DCF to determine by interpolation the amount of ROS produced by 10⁶ cells. Data are displayed as mean $\pm$ SEM.

RT-PCR analysis

Total RNA was isolated from HL-60 cells, differentiated HL-60 cells and human neutrophils using a monophasic solution of phenol and guanidine isothiocyanate (Trizol Reagent, Invitrogen). Total RNA was quantified using an UV spectrophotometer (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Next, the RNA was treated with DNase I to eliminate genomic DNA before carrying out the RT reaction. For the RT reaction, 5 μ g of RNA was incubated at 42°C with 50 μ M Oligo (dT)_{12–18}, 200 U of SuperScript II reverse transcriptase and 40 U/ μ l RNase OUT in a final volume reaction of 20 μ l. PCR analysis was carried out using 2 μ l of the RT reaction in a 50 μ l reaction volume. The PCR protocol used consisted in DNA denaturing at 94°C for 60 s, annealing at 65°C for 30 s (decreasing 0.3°C during each cycle) and extension at 70°C for 30 s. This protocol was carried out for 35 cycles including an initial 5-min denaturation period at 94°C and a final 7 min extension period at 72°C in a thermal cycler 2720 (Applied Biosystems, Carlsbad, CA, USA). Primer sets were designed based on the GenBank sequence for the human P2X₇ receptor (accession no. NM_002563.4): sense oligo 5' ²⁰GGGAGGGGGCTTGCTGTGG³⁸ 3' and antisense oligo 5' ⁴⁴⁸ACCGCTGCTCTTGGCCTTCTG⁴²⁸ 3'. The predicted length for the P2X₇R PCR product was 429 bp. The GAPDH gene (457-bp length product) was used as a positive control. As a negative control, the reverse transcription step was omitted before the PCR analysis was carried out.

Plasma membrane P2X₇R staining

Human neutrophils and HL-60 cells were placed on poly-L-lysine-coated 12-mm glass coverslips for 30 min. Cells were rinsed once with phosphate-buffered saline 1× and fixed with methanol at −10°C for 5 min. Air-dried coverslips were rinsed twice with Tris-buffered saline (TBS, pH7.6) and incubated during 1 h with a polyclonal antibody anti-P2X₇ conjugated with fluorescein isothiocyanate (FITC; 1:100 dilution; Alomone Labs) in TBS supplemented with BSA 1%, pH7.6. After washing periods with TBS, the cells were placed in the stage of a fluorescence microscope (Leica DMLS) and pictures were taken using a Leica DC180 camera. Positive control experiments were carried out using HEK-293 cells stably transfected with mouse P2X₇R and mouse macrophage J774 cells that endogenously express P2X₇R.

Western blot analysis of P2X₇R

Human neutrophils isolated in the presence or absence of apyrase, non-differentiated or differentiated HL-60 cells, HEK-293, P2X₇-transfected HEK-293 and J774 cells were lysed by sonication (15 s, 20 power output) with RIPA lysis buffer supplemented with a protease inhibitor cocktail (Santa Cruz Biotechnology, Inc.) and incubated at 4°C for 30 min. Lysates were centrifuged at 10,000×g for 10 min and supernatants were recovered. Total protein concentration was determined by micro-BCA protein assay (Sigma-Aldrich). Then, 50 µg of total protein was mixed with Laemmli 2× buffer and heated at 95°C for 10 min. Total protein was subjected to 10% SDS-PAGE and electrophoretically transferred to 0.2 µm PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween (TBST). The membranes were probed overnight at 4°C with rabbit polyclonal Ab directed against an intracellular (residues 576–595) or extracellular (residues 136–152) segment of P2X₇R, with or without pre-incubation for 1 h with control peptide Ag (1 µg of peptide per 1 µg of Ab). After washing with TBST, the membranes were incubated with goat anti-rabbit polyclonal HRP-conjugated Ab (1:5,000) in TBST+5% nonfat dry milk during 1 h and 30 min. After subsequent washes, bands were visualised by chemiluminescence (Pierce Rockford, IL, USA) followed by autoradiography.

Results

Functional assay indicates that P2X₇ receptors are absent from plasma membrane

After stimulation with ATP, P2X receptors open a conductive pathway that is permeable to small cations such as Na⁺,

K⁺ and Ca²⁺ and to large cations such as NMDG⁺ and TEA⁺ [33, 38]. It has been reported that stimulation of human neutrophils and HL-60 cells with BzATP increases the intracellular Ca²⁺ concentration and that calcium enters the cell via P2X₇R [42]. If human neutrophils express P2X₇R or any other P2X receptor in their plasma membrane, then activation of these receptors with ATP can be recorded using patch clamp—a highly sensitive technique [20]. When the patch-clamp technique is used in the whole cell configuration, currents from all activated channels located in the plasma membrane can be directly measured. Thus, to evaluate the currents generated by stimulation of human neutrophils with ATP or BzATP (an agonist relatively more selective for P2X₇R), we used the whole cell configuration of the patch-clamp technique. However, both agonists failed to induce any current in neutrophils isolated either in the absence or in the presence of apyrase. Figure 1A, B illustrates the lack of currents at −80 mV from neutrophils isolated in the absence of apyrase bathed and dialyzed with solutions containing Na⁺ and then exposed to 100 µM BzATP (*n*=4) or 250 µM ATP (*n*=8),

Fig. 1 Purinergic stimulation does not induce development of currents in both neutrophils and HL-60 cells. **A** Representative (*n*=4) current trace shows that no currents were induced after stimulating a human neutrophil with 100 µM BzATP. **B** No current was induced by stimulation of neutrophils with 250 µM ATP (*n*=8). Currents were recorded using solutions containing 140 mM Na⁺. **C** Absence of ATP-activated current in a human neutrophil isolated in the presence of 2 U of apyrase (*n*=6). **D** Pretreatment with 1 µM fMLP during 10 min at 37°C of human neutrophils isolated with apyrase did not result in currents after stimulation with 5 mM ATP (*n*=3). **E** Representative current trace (*n*=3) shows that in HL-60 cells, no currents were induced by stimulation with 100 µM BzATP. In this experiment, main cations were K⁺ (115 mM KCl) in the internal solution and TEA⁺ (140 mM TEACl) in the external solution. **F** Rapidly activating current recorded after stimulation of J774 cells with 100 µM BzATP (*n*=9). Grey bars above each trace indicate duration of purinergic stimulation. All currents were recorded at −80 mV. The voltage protocol used is schematically represented in the inset of **A**

respectively. As mentioned above, stimulation with 5 mM ATP ($n=2$) failed to induce whole cell currents in neutrophils isolated in the presence of apyrase (Fig. 1C). It has been proposed that activation of neutrophils with the chemo-attractant *N*-formylmethionyl-leucyl-phenylalanine (fMLP) induced the appearance of purinergic responses [9, 43]. Such activation could induce trafficking of purinergic receptors to plasma membrane as was suggested by Gu et al. [19]. To test this hypothesis, isolated neutrophils were treated with 1 μ M fMLP during 10 min at 37°C and then subjected to patch clamp to measure the ATP-activated currents. Figure 1D shows that treatment with fMLP did not induce the appearance of ATP-activated currents, although neutrophils were activated. Cell activation was visually judged by changes in cell morphology (data not shown). Patch-clamp recordings were also conducted in HL-60 cells stimulated with different ATP concentrations (30, 100 and 250 μ M) or BzATP (100 μ M); however, both agonists failed to induce currents. Figure 1E shows a typical current record from an unresponsive HL-60 cell exposed to 100 μ M BzATP. In contrast, Fig. 1F shows that 100 μ M BzATP induced an inward current at -80 mV in J774 cells ($n=4$) bathed in a solution containing NaCl and dialyzed with an internal solution containing TEACl. Since P2X₇R are permeable to large monovalent cations such as TEA⁺ or NMDG⁺, additional experiments were performed in human neutrophils ($n=4$) bathed and perfused with solutions containing 140 mM TEACl and then stimulated with 100 μ M BzATP; however, this manoeuvre resulted in no TEA⁺ currents at -80 mV (data not shown). We also look for purinergic-dependent Ca²⁺ permeability in neutrophils bathed in solutions containing either low (0.5 mM) or high (20 mM) Ca²⁺. Neutrophils were clamped at -80 mV and then stimulated with 100 μ M BzATP or 250 μ M ATP. Even under high external [Ca²⁺], neutrophils show no currents when stimulated with BzATP or ATP (data not shown, $n=4$ for each agonist).

In DMSO-differentiated HL-60 cells and HL-60 cells, prolonged stimulation of P2X₇R produce a large uptake of impermeable markers such Lucifer Yellow or YO-PRO [42] presumably via pannexin-1 hemichannels [36]. Based on the lack of cationic currents in human neutrophils and HL-60 cells after stimulation with external ATP or BzATP, we hypothesised that treating neutrophils, HL-60 or differentiated HL-60 cells with ATP would result in no ethidium uptake, a fluorescent dye used to assay the large pore formation after P2X₇ receptor activation [33]. As expected, Fig. 2 shows that human neutrophils (a), HL-60 cells (b) and differentiated HL-60 cells (c) did not display ethidium uptake when stimulated with 5 mM ATP. In contrast, P2X₇-transfected HEK-293 cells displayed rapid ethidium uptake upon stimulation with 5 mM ATP (Fig. 2D) since these cells endogenously express pannexin-1. Ethidium

Fig. 2 Lack of ATP-induced ethidium uptake in human neutrophils, undifferentiated HL-60 cells and differentiated HL-60 cells. **A–D** Time course of the fluorescence signals resulting from ethidium uptake reported in arbitrary units (AU), from four different representative cells before and after stimulation. Purinergic stimulation did not induce ethidium uptake in human neutrophils (**A**) ($n=6$), HL-60 cells (**B**) ($n=8$) and differentiated HL-60 cells (**C**) ($n=8$). HEK-293 cells transfected with mP2X₇R (**D**) ($n=5$), used here as a positive control, displayed large fluorescence increase, indicating ethidium uptake upon stimulation with ATP. Grey bars indicate stimulation with 5 mM ATP

uptake was also measured in J774 cells stimulated with ATP (data not shown).

Lack of ROS production in human neutrophils and differentiated HL-60 stimulated by BzATP

Neutrophils kill invading microorganisms by producing large amounts of ROS [29]. It has been suggested that ROS production induced by BzATP in human neutrophils and DMSO-differentiated HL-60 cells is in part mediated by P2X₇R activation in a Ca²⁺-dependent manner [42]. However, the data described above strongly argue against this possibility since we did not detect the presence of functional P2X₇R in these cells. To test this idea, we measured the production of ROS induced after stimulation of neutrophils or differentiated HL-60 cells with BzATP. In agreement with our hypothesis, human neutrophils did not increase the amounts of ROS above resting levels after stimulation with 100 μ M BzATP either in the presence (A in Fig. 3) or absence of external Ca²⁺ (B in Fig. 3). In contrast, when human neutrophils were exposed to 10 μ M PMA—a phorbol ester that stimulates ROS production by direct activation of PKC and subsequent activation of NADPH oxidase—a large increase in ROS concentration was observed both in the presence and in the absence of external Ca²⁺ (A and B in Fig. 3). This observation indicates that our neutrophil preparation was viable and that the biochemical pathways were properly working. Similar results were obtained using HL-60 cells differentiated with DMSO (dHL-60). C in Figure 3 shows that ROS

Fig. 3 BzATP did not induce ROS production in human neutrophils and differentiated HL-60 cells. Cells ($10^6/\text{ml}$) loaded with DCFH₂-DA were treated with vehicle (control), 100 μM BzATP or 10 μM PMA in solutions containing either 2.2 mM (A) ($n=5$) or 0 mM (B) ($n=11$) extracellular Ca^{2+} . C Lack of ROS production in differentiated HL-60 cells stimulated with 100 μM BzATP in the absence of extracellular Ca^{2+} ($n=7$). As expected, treatment with PMA (10 μM) induced a large increase in fluorescence signal in all cells

concentration was not different in the absence or presence of 100 μM BzATP. In contrast, PMA (10 μM) increased ROS concentration to about twice as that observed in control conditions.

P2X₇R mRNA is present in HL-60, differentiated HL-60 cells and neutrophils, but the receptor is not detected at the plasma membrane

To further confirm the absence of P2X₇R in human neutrophils, we examined the expression of mRNA and protein. Expression of P2X₇R mRNA in human neutrophils, HL-60 cells and differentiated HL-60 cells was determined by RT-PCR. Figure 4A shows mRNA expression in neutrophils (lane N), dHL-60 (lane dH) and HL-60 (lane H) cells. A PCR product of 429 bp in length was clearly detected for neutrophils and HL-60 cells. In the case of dHL-60 cells, mRNA expression appeared to be weak. Negative controls (without RT) for these experiments indicate that the observed bands are indeed the result of mRNA expression and not the expression of genomic DNA (data not shown). PCR products of 457 bp that correspond to amplification of the GAPDH gene are shown in the right half of the gel as positive controls.

Since the RT-PCR results indicated the presence of P2X₇R transcripts, we then look for protein expression at the plasma membrane using a FITC-labelled antibody against P2X₇R. This antibody recognises an epitope corresponding to residues 136–152 that are located on the

extracellular loop of the protein. Neutrophils and HL-60 cells were incubated for 1 h with the antibody before the fluorescence labelling was determined. In agreement with the electrophysiological results, neither neutrophils (Fig. 4B) nor HL-60 cells (Fig. 4C) were labelled by the antibody. The three cells labelled in Fig. 4B (indicated by arrows) are most likely basophiles given the shape of their nuclei. In contrast, P2X₇R-transfected HEK-293 (Fig. 4D) and J774 cells (Fig. 4E) were strongly labelled by the antibody. Negative cross-reactivity of the antibody was confirmed using HEK-293 cells transfected with mP2X₄R (data not shown).

Since cell membrane immunostaining experiments gave negative results, we used Western blot as an alternative assay to determine protein expression in whole cells. Total protein was isolated from neutrophils or non-differentiated or differentiated HL-60 cells disrupted by sonication. This approach should be able to detect the protein even if P2X₇R are stored in intracellular organelles as previously suggested [19]. Western blots were revealed using two antibodies, one that recognised (residues 576–595) the intracellular COO[−] terminus (P2X₇-int) whilst the other recognised (residues 136–152) part of the extracellular loop (P2X₇-ext) of P2X₇R. Figure 4F shows that P2X₇R were not detected by either P2X₇-int (a) or P2X₇-ext (b) antibodies in human neutrophils (lane N) isolated in the presence of apyrase (representative of four different volunteers), in differentiated HL-60 cells (lane dH), in non-differentiated HL-60 cells (lane H) and in un-transfected HEK-293 cells as negative control (lane HEK-293, $n=3$). Negative results were also obtained using human neutrophils isolated in the absence of apyrase (data not shown). In contrast, 75-kDa bands corresponding to the P2X₇R were detected by both antibodies in HEK-293 cells transiently transfected with mP2X₇R (Fig. 4F, lane P2X₇ HEK-293, $n=3$) and in J774 cells (Fig. 4F, lane J774, $n=3$) a cell line that endogenously express P2X₇R. Specificity of P2X₇R antibodies was confirmed using the corresponding control antigen peptide. These data show that the P2X₇R protein is not present in whole human neutrophils or in HL-60 cells.

Discussion

In this work, we used RT-PCR, immunofluorescence labelling and Western blotting to assay the presence of mRNA as well as the expression of membrane or intracellular P2X₇R protein in neutrophils and HL-60 cells. In addition, we used the patch-clamp technique to directly measure functional P2X₇R activity at the plasma membrane and carried out spectrofluorometric measurements of ROS production as well as of ethidium uptake to evaluate downstream responses resulting from the activation of

Fig. 4 Lack of P2X₇R protein expression in neutrophils and HL-60 cells. **A** Detection of mRNA in human neutrophils (lane N), differentiated HL-60 cells (lane dH) and HL-60 cells (lane H). GAPDH was used as a positive control. The expected length of the PCR products obtained in these reactions is indicated next to the arrows. **B**, **C** P2X₇R was not detected by antibody P2X_{7-ext} at the surface of human neutrophils (**B**) (representative of $n=3$) or of HL-60 cells (**C**) ($n=2$). White arrows in **B** indicate three cells that are probably contamination and were labelled by the antibody. **D**, **E** Representative pictures of mP2X₇-transfected HEK-293 ($n=4$) and J774 ($n=2$) cells used as positive controls for surface labelling of P2X₇R. All cells were incubated with anti P2X₇-FITC antibody (dilution 1:100) during 1 h as described in “Materials and methods”. **F** Western blots to determine P2X₇R protein in total lysates of human neutrophils (lane N), differentiated HL-60 cells (lane dH), non-differentiated HL-60 cells (lane H), non-transfected HEK-293 cells (lane HEK-293), mP2X₇R-transfected HEK-293 cells (lane P2X₇ HEK-293) and J774 macrophages (lane J774). Neither the P2X_{7-int} antibody nor the P2X_{7-ext} antibody detected P2X₇R protein in human neutrophils ($n=4$), in non-differentiated ($n=4$) or differentiated HL-60 cells ($n=4$) or in non-transfected HEK-293 ($n=3$). In contrast, in HEK-293 transfected with mP2X₇R ($n=3$) and J774 ($n=3$) cells, the mP2X₇R protein was readily detected. GAPDH served as load marker

P2X₇R. Of these techniques, only RT-PCR reported the presence of P2X₇R mRNA; however, this could be the result of contamination with other cells of our neutrophil preparation as suggested by the immunostaining data. In agreement with this idea, Vaughan et al. [44] reported P2X₇R mRNA in neutrophil preparations contaminated with peripheral blood mononuclear cells. Our electrophysiological experiments show lack of ionic currents through P2X₇R in neutrophils or HL-60 cells stimulated with either BzATP or ATP. This was surprising since other groups have

reported the presence of mRNA for P2X₇R in neutrophils and HL-60 cells [8, 42], although other groups reported the presence of mRNA for P2X₁, P2X₄ and P2X₅ receptors, but not for P2X₇ in neutrophils [31, 44]. If these receptors were present in the plasma membrane, they should form aqueous pores selective for cations such as Na⁺ or Ca²⁺ that would open after stimulation with BzATP or ATP. However, we failed to observe any cationic current under different ionic conditions used. The lack of ionic currents upon stimulation was not due to down-regulation of plasma membrane

receptors by ATP released during the hypotonic shock [21] since we also failed to observe purinergic-dependent currents or P2X₇R protein in neutrophils isolated in the presence of apyrase. Finally, we have previously shown that P2X₇R Na⁺ current is sustained during an ATP application lasting more than 30 s, suggesting that this receptor do not desensitise [7] as has been previously shown by others (see [33] for a review). As expected, control experiments carried out using J774 cells or HEK-293 cells expressing mP2X₇R showed large whole cell currents and ethidium uptake (see also [7, 38]), indicating that our recording conditions were optimal for P2X₇R activation. Also, immunofluorescence labelling experiments demonstrate the absence of P2X₇R at the cell surface in neutrophils, and this result is in agreement with our electrophysiological data. Thus, we concluded that P2X₇R are not present in the interior or plasma membrane of human neutrophils.

Our data support several pieces of previously published evidence which questioned the presence of P2X₇R in neutrophils either at the plasma membrane or in intracellular organelles. Some groups reported lack of P2X₇R mRNA in neutrophils [31, 44]. Others reported lack of P2X₇R protein in neutrophils treated with LPS [44], even though LPS is known to up-regulate P2X₇R expression in other cell types such monocytes [22]. Labasi et al. [26] show that neutrophils from wild-type or P2X₇R-deficient mice did not change shape and lack L-selectin shedding in response to ATP, suggesting the absence of surface expression of P2X₇R. Other groups have found that differentiated HL-60 cells show expression of P2X₇R, but their function (measured as an increase in intracellular Ca²⁺ after stimulation with ATP) is not changed during development [1]. Finally, it has been reported that ATP by itself is unable to generate ROS in human neutrophils and differentiated HL-60 cells [39, 43] and that P2X₇R are stored in intracellular organelles susceptible for recruitment to plasma membrane after neutrophil activation [19]. In our hands, however, activation of human neutrophils (judged by cell migration and changes in cell morphology) with fMLP did not result in ATP-activated currents. This suggests that neutrophil activation by short applications of fMLP does not result in mobilisation of P2X purinergic receptors to the plasma membrane. Finally, BzATP is able to induce CD62L shedding via P2X₇R in naive and memory B lymphocytes, but not in neutrophils [40]. In agreement with the latter observation, preliminary experiments from our group failed to show CD62L shedding in human neutrophils stimulated with 10 or 100 μ M BzATP during 10 to 30 min (data not shown; see [23] for experimental conditions used).

Thus, our data do not support previous reports showing purinergic responses upon stimulation of fresh isolated neutrophils or HL-60 cells. For example, a recent work

reported the presence of mRNA for P2X₇R, ROS production, Lucifer yellow or YO-PRO uptake and Ca²⁺ increments after cell stimulation with BzATP [42]. Even though our data show the presence of mRNA for P2X₇R, we could not detect ROS production above control levels, ethidium uptake or Ca²⁺ entry via P2X₇R pores. Although stimulation of ROS production through P2X₇R would be advantageous for neutrophils in order to combat bacterial infection, we think that neutrophils do not respond to external ATP via this receptor. The putative purinergic responses reported so far might be mediated by the activation of P2X₇R in cells other than neutrophils. Contamination of neutrophil preparations with other blood cells that express this receptor has been discussed by Vaughan et al. [44] and Sengstake et al. [40]. In our preparation, mRNA for P2X₇R was observed together with occasional P2X₇R-FITC labelling of cells; however, the cells labelled had nucleus clearly different from that of neutrophils. This represents contamination of our preparation, and such contamination would give false positives when assayed with RT-PCR. Nevertheless, Western blot analysis and functional assay carried out using the patch-clamp technique indicated that at the single cell level, there are no P2X₇R present. We do not have an explanation for mRNA expression but lack of P2X₇R protein in our HL-60 cells or for the evidence in favour of mRNA and protein expression reported by other groups. We wonder if this difference may somehow be related to different batches of HL-60 cells used among different groups.

Pannexin-1 is a membrane protein that forms large pores permeable to fluorescent dyes such as ethidium [36]. This protein is activated downstream of P2X₇R; thus, it has been proposed that prolonged activation of P2X₇R activates pannexin-1, and this large pore causes cell death. Using RT-PCR, we also investigated the presence of pannexin-1 mRNA in neutrophils, HL-60 and differentiated HL-60 cells (data not shown). Our RT-PCR experiments show lack of pannexin-1 mRNA, thus supporting the result showing lack of ethidium uptake in neutrophils and HL-60 cells. Thus, based on the absence of P2X₇R at the plasma membrane together with the absence of pannexin-1 mRNA, we propose that large pores are not formed in human neutrophils and HL-60 cells and that death of these cells via pannexin-1 cannot happen. This result is in agreement with data recently published which show that stimulation of human neutrophils with external ATP resulted in long-lived neutrophils via the activation of P2Y₁₁ receptors [44].

In summary, our data support the contention that P2X₇R are not present in the plasma membrane of human neutrophils and of HL-60 cells. It remains to be determined whether other P2X receptor subtypes are expressed in intracellular organelles and thus can serve as transducers of the external ATP signal. We argue that the putative

physiological responses and/or ion fluxes after purinergic stimulation previously reported are not mediated by P2X₇R, and hence, P2X₇R involvement in neutrophil function should be reconsidered.

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