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P2X7 receptors mediate resistance to toxin-induced cell lysis

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ABSTRACT

In the majority of cells, the integrity of the plasmalemma is recurrently compromised by mechanical or chemical stress. Serum complement or bacterial pore-forming toxins can perforate the plasma membrane provoking uncontrolled Ca^{2+} influx, loss of cytoplasmic constituents and cell lysis. Plasmalemmal blebbing has previously been shown to protect cells against bacterial pore-forming toxins. The activation of the P2X7 receptor (P2X7R), an ATP-gated trimeric membrane cation channel, triggers Ca^{2+} influx and induces blebbing. We have investigated the role of the P2X7R as a regulator of plasmalemmal protection after toxin-induced membrane perforation caused by bacterial streptolysin O (SLO).

Our results show that the expression and activation of the P2X7R furnishes cells with an increased chance of surviving attacks by SLO. This protective effect can be demonstrated not only in human embryonic kidney 293 (HEK) cells transfected with the P2X7R, but also in human mast cells (HMC-1), which express the receptor endogenously. In addition, this effect is abolished by treatment with blebbistatin or A-438079, a selective P2X7R antagonist. Thus blebbing, which is elicited by the ATP-mediated, paracrine activation of the P2X7R, is part of a cellular non-immune defense mechanism. It pre-empts plasmalemmal damage and promotes cellular survival. This mechanism is of considerable importance for cells of the immune system which carry the P2X7R and which are specifically exposed to toxin attacks.

1. Introduction

Serum complement or bacterial pore-forming toxins perforate the plasma membrane of a host cell and cause influx of extracellular — and efflux of cytoplasmic components, which might lead to cell death [1–4]. It is thus not surprising that eukaryotic cells have developed membrane repair mechanisms and adapted them to suit the nature and severity of plasmalemmal injury [5,8]. A breakdown of the Ca^{2+} concentration gradient between cytoplasm and extracellular milieu serves as an indicator of plasmalemmal disruption and acts as molecular guidance for the intracellular repair machinery. Intracellular [Ca^{2+}] elevations are recognized by Ca^{2+} sensors such as proteins of the annexin family, which bind Ca^{2+}-dependently to negatively-charged phospholipids [9] and have been ascribed a role in plasmalemmal repair [10,13].

Blebbing – a temporary detachment of the lipid bilayer from the submembranous cytoskeleton – is considered to be an early sign of intracellular Ca^{2+} elevation, which indicates imminent or incipient membrane injury [14]. We have recently proposed blebbing to be a damage-control mechanism, which is triggered after the failure of plasmalemmal resealing [15]. The ability to bleb provides a clear benefit of survival for a cell confronted with attacks on its plasmalemma by pore-forming toxins [15].

Since P2X7R activation is associated with an increase in membrane blebbing [16], we have investigated the role of this receptor as a molecular regulator of plasma membrane protection after plasmalemmal perforation caused by the bacterial toxin SLO. The ionotropic P2X7R has two transmembrane domains; intracellular NH_2- and COOH-termini and forms a homotrimer [17]. It is ubiquitously expressed, with high levels in immune cells and exists as a multiprotein complex, including the non-muscle myosin heavy chains and other cytoskeletal elements [18,19]. The activation of the P2X7R by extracellular ATP opens a cationic channel which gradually dilates to a larger pore [20,22]. It is itself subject to regulation by numerous polymorphic variants and isoforms which increase or decrease its efficiency [23]. In macrophages primed by lipopolysaccharide, channel opening is additionally associated with the secretion of pro-inflammatory cytokines IL-1β [24,25] and IL-18 [26,27]. P2X7R activation is followed by downstream effects such as blebbing [28,29], microvesicle shedding [30,31], cell fusion [32], proliferation [33] and eventually cell death [34]. Furthermore, in the absence of ATP, P2X7R plays a role in recognition and phagocytosis of foreign particles [35,36].

We demonstrate that the expression of the P2X7R confers resistance to the attack by bacterial pore-forming toxins. This protective effect can be increased by activation of the P2X7R with ATP and is abolished by

Abbreviations: HEK, human embryonic kidney 293 cells; HMC-1, human mast cell line 1; P2X7R, P2X7 receptor; RT, room temperature; SLO, streptolysin O

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pretreatment with blebbistatin or A-438079, a selective P2X7R antagonist. We further suggest that the paracrine activation of this receptor by ATP, released by damaged neighboring cells, initiates blebbing and thus induces resistance to toxin-induced cell lysis. Blebbing is a characteristic feature of injured cells. It is thus conceivable that cells – in particular within inflammatory foci – use this ATP-triggered, P2X7R-mediated warning system to alert their neighbors to an approaching wave of bacterial toxins.

2. Materials and methods

2.1. Cell culture

HEK cells were maintained in DMEM (Dulbecco's modified Eagle's medium), containing 2 mM glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS) and were transfected as described [11,37]. HEK cells stably transfected with the rat P2X7R (HEKrtP2X7) or the human P2X7R (HEKhuP2X7) [38] were cultured in DMEM/F12 medium supplemented with 2 mM glutamax, G418 (300 μg/ml), 2 mM MgCl2 and 10% FCS. HMC-1 were cultured in IMDM with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin as described [39]. All cells were grown in 5% CO_2 at 37 °C in a humidified incubator. For the viability experiments, the cells were challenged with 750 U/ml SLO from *Streptococcus pyogenes* (Sigma-Aldrich, Buchs, Switzerland) in Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES; pH = 7.4) containing 2.5 mM $CaCl_2$ or 1 mM EGTA (for the experiment without extracellular calcium) for 15 min at room temperature (RT). Cell viability was quantified by the cell health indicator alamarBlue® following the manufacturer's instructions (Life Technologies, Carlsbad, USA [40]). To inhibit P2X7R-specific ATP stimulation, the cells were incubated with 1 μM or 10 μM A-438079 (Sigma-Aldrich, Buchs, Switzerland) for 15 min at RT.

2.2. Expression of annexins as fusions with fluorescent proteins

The coding sequences of annexins A1 and A2 were cloned into the Living Colors Fluorescent protein vectors pEGFP-N1 or pEYFP-N1 (Clontech, Saint-Germain-en-Laye, France) following PCR amplification from human bladder smooth muscle cDNA. The fluorescent protein was attached to the C-terminus of the annexins, which has been shown not to affect the protein function [41]. The oligonucleotide pairs used for PCR correspond to the N-termini (forward) and C-termini (reverse, lacking the stop codon) of annexins and contain appropriate restriction sites to allow the in-frame cloning with fluorescent proteins.

For annexin A1, 5′-TTTAAGCTAGCG**ATG**GCAATGGTATCAGATTCCTC-3′ forward and 3′TTATTCTGCAGGTTTCCTCCAAACAGAGCCAC-5′ reverse.

For annexin A2, 5′-AATATAGCTAGC**ATG**TCTACTGTTCACGAAATCCTG-3′ forward and 5′ATAAACTCGAGTTAGTATAGGCTTTGACAGAC-3′ reverse.

2.3. Imaging

Glass coverslips with annexin-expressing HEK cells were mounted in a perfusion chamber at RT in Tyrode's buffer containing 2.5 mM $CaCl_2$. To induce blebbing, the ATP analog 2′(3′)-O-(4-Benzoylbenzoyl)ATP (BzATP) was added to the cells at concentrations between 10 μM and 100 μM (in Tyrode's buffer). Cell lysis was monitored by irreversible elevation of intracellular [Ca^{2+}] above 20 μM using permanent plasmalemmal translocation of annexin A1 as a read-out [15]. Cells were challenged with 750 U/ml SLO in Tyrode's buffer for 15 min at RT. When indicated, the cells were pretreated with blebbistatin (100 μM, 30 min) and/or ATP (30 μM, 10–15 min) before the addition of SLO. The fluorescence was recorded in an Axiovert 200 M microscope with a laser scanning module LSM 510 META (Carl Zeiss AG, Feldbach, Switzerland) using a 63× oil immersion lens. The images were analyzed using the 'Zeiss LSM Image examiner' software package (Carl Zeiss AG). To determine the percentage of lysis, the number of cells with annexin A1 translocation was divided by the total cell number [15]. Transient plasmalemmal translocations of fluorescently-labeled annexin A2 were used to monitor elevations of intracellular Ca^{2+} in the low micromolar range [11,42].

Numerical data are expressed as mean values together with the standard error. The statistical analyses were performed using GraphPad Prism 5.04 and Microsoft Excel 2010. The level of significance was set at $P < 0.05$. Significant differences are marked with asterisks.

2.4. Western blotting

HEK, HEKrtP2X7 and HEKhuP2X7 cells were resuspended in SDS sample buffer (200 mM Tris–HCl, 40 mM EGTA, pH 7, 3.1% SDS, 7.7% β-mercaptoethanol, 13.4% glycerol and 60 μg/ml of bromophenol blue) and boiled for 1 min. Samples were run on 8% SDS–polyacrylamide minigels together with a molecular weight marker (Page Ruler Prestained, Thermo Scientific, Erembodegem, Belgium). Equal amounts of protein were loaded for the different protein extracts as estimated by a test gel. Blotting was carried out overnight onto a PVDF membrane (Millipore AG, Zug, Switzerland). Unspecific binding sites were blocked with 4% non-fat dry milk in low salt buffer (0.9% NaCl, 10 mM Tris-Base, 0.1% Tween 20) for 1 h at RT. Primary antibodies (polyclonal rabbit-anti-P2X7R, Alomone Labs, Jerusalem, Israel) were diluted in low salt buffer supplemented with 2% non-fat milk powder and incubated for 1 h at RT. The blot was washed 3 times with low salt buffer for 10 min. Secondary antibodies (HRP-donkey-anti-rabbit IgG, GE Healthcare, Glattbrugg, Switzerland) were diluted in low salt buffer supplemented with 2% non-fat milk powder and incubated for 45 min at RT. Finally, the blot was washed again 3 times with low salt buffer for 10 min. Chemiluminescence reaction was performed using Western Bright ECL from Advansta (Menlo Park, CA, USA) and the signal was detected by a Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France).

3. Results

In order to investigate P2X7R function in plasmalemmal repair, we initially applied a well characterized model system (HEK cells), in which blebbing has previously been associated with increased survival [15]. We confirmed that the transcription (Fig. 1A) and protein expression (Fig. 1B) of P2X7R occurred in HEK cells stably transfected with the P2X7R gene, but not in control cells. Immunostaining with antibodies against the P2X7R demonstrated that it was localized to the plasmalemma, as previously described, whereas untransfected HEK cells were devoid of signal (Fig. 1C).

The activation of a functional P2X7R leads to an increase in [Ca^{2+}], followed by blebbing [43,44]. Therefore, the functionality of the P2X7R in stably transfected HEK cells was established by stimulation with increasing concentrations of BzATP [45] and by simultaneous monitoring of blebbing activity and [Ca^{2+}] by Ca^{2+}-dependent plasmalemmal translocation of fluorescently labeled annexin A2 [11].

HEKrtP2X7 cells responded to increasing concentrations of BzATP by an increased plasmalemmal blebbing (Fig. 2A). After stimulation with 10 μM BzATP, 2.4 ± 1.8% of the cells blebbed, whereas at 30 μM BzATP, ~50% of the cells displayed blebbing during 1 h of treatment. At a concentration of 100 μM BzATP, almost all cells blebbed (98.6 ± 1.4%; 450 cells on 30 slides, Supplementary Movie 1).

Annexin A2 reversibly translocates between the cytoplasm and the plasmalemma at low micromolar $[Ca^{2+}]_i$ [42]. In non-stimulated cells, annexin A2 localizes within the cytoplasm [11]; this pattern was not altered by the expression of the P2X7R. Blebbing closely correlated with annexin A2 membrane translocation, confirming Ca^{2+} influx through the opened P2X7 channel after stimulation with BzATP (Fig. 2B). Stimulation with 30 μM BzATP led to increased blebbing (Fig. 2C) and plasmalemmal translocation of annexin A2 (Fig. 2D) in 64.5 ± 8.1% of cells (495 cells on 33 slides).