Pannexin1 is part of the pore forming unit of the P2X7 receptor death complex

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\begin{abstract}
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The purinergic receptor P2X\textsubscript{7} is part of a complex signaling mechanism participating in a variety of physiological and pathological processes. Depending on the activation scheme, P2X\textsubscript{7} receptors in vivo are non-selective cation channels or form large pores that can mediate apoptotic cell death. Expression of P2X\textsubscript{7}R in \textit{Xenopus} oocytes results exclusively in formation of a non-selective cation channel. However, here we show that co-expression of P2X\textsubscript{7}-R with pannexin1 in oocytes leads to the complex response seen in many mammalian cells, including cell death with prolonged ATP application. While the cation channel activity is resistant to carbenoxolone treatment, this gap junction and hemichannel blocking drug suppressed the currents induced by ATP in pannexin1/P2X\textsubscript{7}-R co-expressing cells. Thus, pannexin1 appears to be the molecular substrate for the permeabilization pore (or death receptor channel) recruited into the P2X\textsubscript{7}-R signaling complex.

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\end{abstract}

1. Introduction

Extracellular ATP plays an important signaling role in numerous and varied physiological processes. In the CNS, this nucleotide has been proposed to modulate neuronal and glial activity \cite{1}. ATP also controls peripheral blood flow \cite{2}. ATP signaling is mediated by two types of purinergic receptors, the metabotropic P2Y receptors and the ionotropic P2X receptors \cite{3}. Ionotropic P2 receptors respond to the ligand ATP or related agonists by opening small non-selective cation channel. For certain P2X receptors, notably P2X\textsubscript{7}-R \cite{4} repeated or prolonged application of the ligand leads to the formation of a pore large enough to allow influx of tracer molecules typically used to assess gap junction function. Prolonged activation of the receptor can lead to cell death.

Two hypotheses have been presented to explain the conversion of the non-selective cation channel to the cytolytic pore \cite{5}. The pore may form by accretion of the channel forming subunits to a larger multimer resulting in pore dilation. Alternatively, pore formation may require additional components. Attempts to reconstitute the pore formation in exogenous expression systems have been unsuccessful, favoring the second hypothesis. While pore formation can be observed in some cell types expressing P2X\textsubscript{7}-R, other cells, including \textit{Xenopus} oocytes, exhibit only the cation channel activity when the P2X\textsubscript{7}-R is expressed \cite{6}. Interestingly, while pore formation could not be reconstituted in oocytes with the P2X\textsubscript{7}- receptor alone, pore formation was observed in response to injection of the cells with macrophage mRNA \cite{7}. These observations are highly suggestive for the need of an additional component for pore formation.

We have noticed that the properties of the P2X\textsubscript{7}-R pore match those of pannexin1 channels in several respects. Common properties include permeability for large molecules and dyes typically used to analyze gap junction channels, sensitivity to cytoplasmic acidification, single channel conductance, and sensitivity to gap junction channel blockers \cite{8–11}.

We consequently tested whether pannexin1 could represent the P2X\textsubscript{7}-R pore by manipulating pannexin1 expression in a human astrocytoma cell line (1321N1 cells) stably expressing the rat P2X\textsubscript{7}-R and by expressing human pannexin1 and human P2X\textsubscript{7}-R (in isolation and in combination) in \textit{Xenopus} oocytes. Through the use of a variety of approaches (fluorescence imaging, electrophysiological recordings, and Western blotting) we provide evidence that pannexin1 channels are likely the pore forming units activated by ATP stimulation of the P2X\textsubscript{7}-R.

2. Materials and methods

\textit{Cell culture.} The human astrocytoma cell line (1321N1) stably expressing the rat-P2X\textsubscript{7} receptor (r-P2X\textsubscript{7}-R) (construct kindly provided by Dr. AnnMarie Surprenant, Institute of Molecular Physiology, University of Sheffield, UK) was grown in DMEM (Gibco) containing 10% FBS (Gibco) and 1% antibiotics, as previously described \cite{8}. Two days before experimentation, cells were plated in glass-bottomed (MatTek) dishes for dye-uptake measurements.

\textit{siRNA.} P2X\textsubscript{7}-R-expressing astrocyoma cells were treated with 1 µg/1.5 ml small interference RNAs corresponding to the human pannexin1 using 6 µl/1.5 ml oligofectamine reagent (Invitrogen). After overnight exposure, transfection reagents were removed and cells incubated for 30 h in DMEM-FBS medium. The target sequences for the siRNAs were: (1) GCCUCCUUUGUGGAUUCAt and (2) GGAGAUCUGCAUUAGCUAAtt for human pannexin1. Target sequences for mouse pannexin1 were: (1) GCCUCCUUUGUGGAUUCAt, (2) GGAAACUUUGCAAAAGCUAAtt, and (3) GGUCUGGAGAACAUUAAAtt. Control siRNA “Silencer™ Negative” 1-3 from Ambion Inc. (Austin, TX, USA) were used as controls.
Dye uptake. r-P2X7R expressing 1321N1 cells were bathed for 5 min in a low calcium solution containing 800 μM MgCl₂, 100 μM EGTA, pH 7.3, 100 μM BzATP, and 5 μM YoPro. Dye fluorescence intensity was measured following 5 min stimulation. After background subtraction, Yo-Pro fluorescence intensity was normalized to values obtained from cells bathed in low calcium solution containing 5 μM YoPro. Dye fluorescence was captured using an Orca-ER CCD camera attached to an inverted Nikon microscope equipped with a 20x dry objective and 488 nm filter set using Metaffer software.

Membrane blebbing. To visualize membrane blebbing induced by P2X7R activation, 1321N1 astrocytoma cells plated on MatTek dishes were exposed to the same low divalent cation solution described above for dye uptake measurements containing the amphiphilic styryl dye FM4-64 (1 μM final concentration; Molecular Probes). Changes in cell membrane morphology (blebs) induced by 150 μM BzATP, in the absence and presence of 50 μM carbenoxolone, were acquired at 1 Hz over a period of 2–3 min following the addition of BzATP using an Orca-ER CCD camera attached to an inverted Nikon microscope equipped with a 40x dry objective and 594 nm filter set using Metafer software. After playback and analysis of the saved images, the number of cells within the field of view displaying membrane blebs was quantified and data expressed as fraction of total number of cells. Three to four independent experiments were performed.

Western blots. After YoPro influx measurements, cells were lysed with 2x Laemmli buffer (containing 100 mM DTT) and subjected to electrophoresis using SDS-PAGE mini-gels (4–20%; BioRad). Proteins were transferred to nitrocellulose membranes (Schleider & Schuell) and after overnight blocking (2% non-dry milk in 1x PBS with 0.05% Tween-Twenty), membranes were blotted using anti-Pannexin1 and anti-GAPDH antibodies (1:2000; Research Diagnostics) primary antibodies for 2 h at room temperature. After several washes with 1XPBS-TweenTwenty solution, membranes were incubated for 2 h with rabbit-anti-chicken, and goat-anti-mouse HRP conjugated secondary antibodies (1:2000). Bands were visualized in X-ray films (Kodak) after incubation with the enhanced chemiluminescent reagent (Amersham). Quantification of pannexin1 expression levels was performed using NIH-imaging software.

Electrophysiology: Preparation of oocytes and electrophysiological recording were performed as described [13]. Human pannexin1 (h-PANX1) was kindly provided by Dr Graeme Bolger, University of Alabama and human P2X7R kindly provided by Dr. Annemarie Suprenant, University of Sheffield, UK. Pannexin1, in Bluescript, was linearized with Kpn I, h-P2X7, in pcDNA3, with Avr II. In vitro transcription was performed with the polymerases T3 (pannexin1), and T7 (P2X7-R) using the Message Machine kit (Ambion). mRNAs were quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked following 5 min stimulation. After background subtraction, Yo-Pro fluorescence intensity was normalized to values obtained from cells bathed in low calcium solution containing 5 μM YoPro. Dye fluorescence was captured using an Orca-ER CCD camera attached to an inverted Nikon microscope equipped with a 20x dry objective and 488 nm filter set using Metafer software.

To test whether Pannexin1 is involved in P2X7R-mediated membrane permeabilization, we used the human astrocytoma cell line 1321N1 stably expressing the rat P2X7R. First, we

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3. Results

We have previously shown that the influx of large tracer molecules following activation of P2X7R was prevented by a series of compounds typically used as gap junction channel blockers [8]. These observations raised the possibility that these gap junction channel blockers could either interfere with the P2X7 receptor itself and/or interfere with an additional channel protein recruited by the P2X7R. A non-connexin candidate that shares similar pharmacological properties to gap junction channels is the pannexin1 channel.

To test whether pannexin1 is involved in P2X7-R-mediated membrane permeabilization, we used the human astrocytoma cell line 1321N1 stably expressing the rat P2X7R. First, we
investigated whether these cells expressed endogenous pannexin1. Western blot analysis indicated that these cells expressed low levels of pannexin1 (Fig. 1a); moreover, 48 h treatment with two small interference RNA (siRNA) targeting human pannexin1 greatly reduced pannexin1 levels (Fig. 1a). Similar results were obtained for primary cultures of mouse cortical astrocytes (Fig. 2). In the next set of experiments, P2X7R expressing astrocytoma cells were treated with the two pannexin1 siRNA sequences to measure BzATP-induced YoPro uptake. As shown in Fig. 1b, downregulation of human pannexin1 by siRNA significantly reduced YoPro uptake compared to untreated cells. These data, therefore, suggest that pannexin1 is likely to contribute to membrane permeabilization that occurs after activation of the P2X7R. That the inhibition by siRNA of pannexin1 protein level was more pronounced than the inhibition of BzATP-induced YoPro uptake can have several explanations. For example, pannexin protein in different cellular compartments may have different turnover rates or channel kinetics may change with expression levels. Alternatively, the residual YoPro uptake may be through unspecific leakage or through a specific, yet unidentified alternate pathway.

To further verify the hypothesis that pannexin1 is part of the P2X7R permeabilization pore, electrophysiological and dye uptake studies were performed in *Xenopus* oocytes expressing the human P2X7R and human pannexin1. As shown in Fig. 3a, ATP (200 μM) induced an inward current in oocytes expressing P2X7R (middle trace), but not in non-transfected oocytes (not shown). Also, as shown previously (11), ATP up to 500 μM does not induce pannexin currents in oocytes expressing pannexin1 only. Co-expression of P2X7R and pannexin1 increased the inward current (bottom trace) as compared to oocytes expressing only the receptor. Quantification of ATP-induced currents indicates that co-expression of the two proteins led to a sixfold increase in inward current (from 0.5 μA in P2X7R to 3 μA in P2X7R + pannexin1; Fig. 3b). The dose–response curve for the P2X7R agonist BzATP is shown in Fig. 3c. The concentration dependence of the currents induced by the agonist was the same in oocytes co-expressing the receptor with pannexin1 as has been documented for the human P2X7R receptor [14]. However, the currents in the co-expressing cells were considerably larger than in oocytes

![Fig. 2. Expression of pannexin1 in astrocytes. Western blot of lysates of primary cultures of mouse cortical astrocytes after treatment with siRNA to three different targets in mouse pannexin1 and with three different control siRNAs were performed. Beta tubulin was used to test for uniformity of loading.](image)

![Fig. 3. Membrane currents from oocytes expressing rat P2X7R or co-expressing the receptor with human pannexin1. (a) The membrane potential was clamped at −50 mV and 5 mV depolarizing pulses were applied (top trace). Application of 200 μM ATP resulted in a considerable inward current. (b) Mean ATP-induced (200 μM) peak membrane currents in oocytes expressing P2X7R alone or in combination with Panx1. (n = 5). (c) Dose–response curve of membrane currents (squares) and membrane conductance (triangle) induced by BzATP. Data were normalized to values obtained with the highest BzATP concentration tested (200 μM).](image)

![Fig. 4. Membrane currents and morphology of oocytes co-expressing P2X7R and Panx1 with prolonged exposure to ATP. (a) Brief exposure to 200 μM ATP resulted in a reversible inward current. Lower exposure to 500 μM ATP resulted in a current that initially reversed partially but subsequently increased to levels that could not be clamped anymore, indicating membrane breakdown. (b) Non-injected control oocytes (upper left quadrant) and oocytes co-expressing P2X7R and Panx1 (lower right quadrant) were exposed to 300 μM ATP. (c) Within 3 min a dramatic change of pigmentation was observed in the co-expressing but not in the control oocytes. (d) After 5 min all co-expressing oocytes sprang leaks with yolk oozing out of the cells. A video clip of this sequence of events is shown in Supplement 1.](image)
expressing only the receptor, as would be expected if activation of P2X7R resulted in the opening of additional, presumably pannexin1 channels.

In cells expressing only P2X7R and in those co-expressing the receptor with pannexin1 the higher concentrations of the agonist resulted in changes of the pigment distribution, most likely as a consequence of the associated calcium influx. Typically, this change remained inconsequential for the membrane integrity of oocytes expressing the receptor alone. In contrast, the co-expressing oocytes exhibited a breakdown of membrane resistance (Fig. 4a) and yolk could be observed oozing from the cells under these conditions (Fig. 4b–d and Supplement 1). It appears that the death signaling observed in macrophages exposed to ATP can be reconstituted in oocytes co-expressing P2X7R and pannexin1. Carbenoxolone delayed the onset of cell death but did not block it. The failure of complete inhibition of cell death probably is due to the high expression levels of the proteins in combination with the inability of carbenoxolone to close pannexin1 channels completely. To further address this issue, P2X7R-expressing 1321N1 astrocytoma cells were tested for an effect of carbenoxolone on cell membrane blebbing induced by BzATP (150 lM). As shown in Supplement 2 carbenoxolone (50 lM) suppressed membrane blebbing, which is an early symptom of cell death.

Fig. 5a shows currents induced by 200 lM ATP in oocytes expressing the human P2X7 receptor. As described by others [15,16] the currents desensitize during ATP application and desensitization outlasts the stimulus as repeated applications of ATP evokes smaller amplitudes of the currents. Inclusion of carbenoxolone (CBX, 100 lM) suppressed membrane blebbing, which is an early symptom of cell death.

Flufenamic acid, another gap junction blocker, also has been shown to inhibit ATP-induced dye uptake in astrocytes [8]. However, in contrast to carbenoxolone, flufenamic acid appears to exert its inhibitory action at more than one target. As shown in Supplement 3, 100 lM flufenamic acid attenuated the ATP-induced currents in oocytes expressing P2X7R alone. In oocytes expressing exclusively pannexin1 flufenamic acid inhibited the voltage-activated pannexin1 channels (Supplement 4). However, higher concentrations (300 lM) were required to observe significant effects. This is in line with the inhibition of pannexin1 channels by flufenamic acid reported earlier [9].

To activate pannexin1 channels by voltage the membrane must be depolarized to positive values [10,17]. However, activation of pannexin1 channels through P2X7R stimulation occurred at the cell’s resting membrane potential. At resting potential, pannexons have been reported to be activated by increases in cytoplasmic calcium concentration [11]. It is therefore feasible that the calcium permeability of the non-selective cation conductance could account for pore formation in oocytes co-expressing pannexin1 and P2X7R. On the other hand, pore formation by P2X7R in macrophages and other cells is reported to be facilitated by low extracellular calcium concentration [6]. To test for extracellular calcium involvement in pannexin1 activation through P2X7R, activation of pannexin1 channels by ATP exposure was studied in calcium-free (no calcium added) oocyte Ringer solution. Removal of extracellular calcium did not abolish pannexon activation through P2X7R, 20 lM BzATP still elicited large currents that were attenuated by carbenoxolone in oocytes co-expressing Panx1 and P2X7R (Fig. 6).

To gather further support for the hypothesis that pannexin1 channels provide membrane permeabilization following P2X7R activation, carboxyfluorescein uptake was evaluated in oocytes expressing the receptor alone and in combination with pannexin1. As indicated in Fig. 7, expression of P2X7R result in smaller amplitude currents. Inclusion of the gap junction channel blocker carbenoxolone did not change this pattern (Fig. 5b), indicating that the drug does not act directly on P2X7R. Co-expression of P2X7R with pannexin1 yielded considerable larger currents in response to ATP stimulation (Fig. 5c). The currents desensitized as in oocytes expressing P2X7R alone. In contrast, in carbenoxolone treated co-expressing cells, the ATP induced currents were attenuated. Fig. 5d shows a quantitative analysis of the carbenoxolone effect.
alone or pannexin1 alone did not lead to dye uptake following agonist stimulation, but when co-expressed with pannexin1, a threefold increase in fluorescence intensity was recorded. Thus, these data support the hypothesis that recruitment of another protein, most likely pannexin1, is necessary for P2X7-R to provide membrane permeability to large molecules.

4. Discussion

P2X receptors are ionotropic receptors that open Ca\(^{2+}\) permeable channels when activated by pyrimidine nucleotides. However, during prolonged exposure to agonists, certain of these receptors (P2X\(_2\), P2X\(_4\), P2X\(_5\), and P2X\(_7\)) can lead to the formation of permeabilizing pores, allowing the passage of large molecules (North, 2002).

Expression of cloned P2X7-R in mammalian cells led to uptake of moderately large fluorescent dyes upon exposure to P2X receptor agonists [4], as had been reported for ATP activation of the permeabilizing pore (formerly known as the P2Z receptor) [18–21]. However, this property has not yet been observed in P2X7-R RNA injected Xenopus oocytes [6,22], although when macrophage mRNA is co-injected, oocyte permeabilization occurs [7]. This finding, together with the observations that the permeability of these “pores” to large molecules, varies among mammalian cell types [5] led to the conclusion that another “factor” was required to provide P2X7-R with the P2Z property. Since then, two distinct hypotheses have been proposed to account for the transformation of P2X7-R permeability from cationic channel to conduit for fluorescent molecule flux that occurs upon prolonged agonist activation. One is based on the idea that incorporation of multiple P2X7-R subunits confer successive increases of permeability to large molecules [23–25]; the other hypothesis considers the existence of another independent channel permeable to large molecules [22,26–28] that is recruited following P2X7-R activation by means of an intracellular signaling cascade [29].

P2X7-R pore formation has been reported to be dependent on the carboxyl terminal cytoplasmic segment of P2X7-R. After removal of this segment, pore formation is lost while the cation permeability remains unaltered [14,30], and several intracellular binding partners have been identified [31], that could provide a link between the receptor itself and the pore. Because pore formation rate is inversely related to extracellular calcium concentration [24,32], it is likely that the influx of calcium is not a crucial factor in pore formation (but see [29]).

Pannexins are a new group of proteins that share low homology with invertebrate gap junction proteins, the innexins [33,34]. When expressed in Xenopus oocytes, pannexin1 and 2 have been reported to form gap junction channels and also to function as hemi-gap junction channels that are sensitive to gap junction channel blockers, including carbenoxolone [9,17]. Gap junction channel formation by pannexins in vivo, however, remains to be demonstrated [35]. The non-junctional pannexin1 channels are voltage sensitive, 400pS channels that are modulated by intracellular calcium and by mechanical stretch [10,11]; these large conductance channels have been proposed to mediate ATP release from erythrocytes [12].

Interestingly, we have previously reported that P2X7 receptors are likely involved in ATP release from astrocytes, and have shown that gap junction channel blockers interfere with P2X7-R activity. [8]. These findings, however, did not discriminate between the possibility that these blockers either interfere with the P2X7 receptor itself or that the P2X7-R mediated effects involved an additional channel protein that is targeted by the blockers. Consistent with the latter, the P2X7 receptor is not a stand-alone channel, but a signaling complex [31]. To discriminate between these possibilities, we tested the effects of the gap junction channel blocker carbenoxolone and FFA on currents in Xenopus oocytes expressing P2X7-R alone or co-expressing the receptor with pannexin1. The results indicate that pannexin rather than P2X7-R is affected by carbenoxolone, while FFA acts on both P2X7 receptor and pannexin.

The involvement of pannexin1 in dye uptake/ATP release is also supported by the interference of pannexin1 specific siRNA in the human astrocytoma cell line 1321N1 stably expressing the rat P2X7-R. Recently, a similar suppression by pannexin1 siRNA was reported for dye uptake in macrophages [36]. However, inexplicably, no current associated with pannexin1 was found in that study. Furthermore, a great portion of the evidence for a pannexin1 involvement was based on a “pannexin mimetic peptide” whose specificity was not documented.

The activation of pannexin1 could occur through protein–protein interaction or through calcium as second messenger. Initial binding of ATP to P2X7 receptors leads to a non-specific cation permeability facilitating the influx of calcium among other cations. Pannexin1 channels can be activated by micromolar cytoplasmic calcium [11]. However, consistent with findings in macrophages, where pore formation is independent of extracellular calcium, it appears that calcium influx is not required for pannexin1 activation through P2X7 receptors. The activation may thus occur through protein–protein interaction. Such an interaction between P2X7- and pannexin1 in macrophages is indicated by the co-immunoprecipitation of the two proteins by a P2X7 antibody [36].

It appears that pannexin1 is promiscuous in its interactions with purinergic receptors. In addition to the activation through P2X7 receptors shown here it can, at least in oocytes, also be activated by ATP through the metabotropic receptors P2Y1 and P2Y2 [11]. Thus, intercellular calcium signaling may involve pannexin1 channels in combination with either P2X7 or P2Y receptors. Calcium wave initiation could be exclusively mediated by pannexin1 due to its channel properties, which include mechanosensitivity and high ATP permeability. Wave propagation on the other hand requires an ATP receptor in

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**Fig. 7. Uptake of carboxyfluoresceine in oocytes.** Non-injected control oocytes, oocytes expressing P2X7-R alone or Pannx1 alone, and oocytes co-expressing P2X7-R and Pannx1 were exposed for 5 min to Ringer solution supplemented with 10 mM carboxyfluoresceine and 30 μM BzATP. Fluorescence was determined on cryosections and was normalized to the values observed in control oocytes.
addition to an ATP release channel. Pannexin1 in combination with either P2Y or P2X7 receptors may serve this function. Both types of receptors when activated by ATP can open pannexin1 channels as shown here for P2X7 and previously for P2Y1 and P2Y2 [11]. The question that remains to be further investigated, however, is why activation of P2X-R but not of metabotropic P2Y-R leads to membrane permeabilization. With the detection of pannexin1 in astrocytes, the list of ATP releasing cells also expressing pannexin1 is growing. The list includes erythrocytes that express this “gap junction protein” without ever forming cell–cell junctions.

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Appendix A. Supplementary data

References