



Article

Rat P2X7 Receptor Show Functional Independence Between Macropore Formation and Scavenger Activity

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Abstract

The purinergic ionotropic receptor P2X7 stands out from other members of the P2X family due to its ability to form a macropore, activate multiple intracellular signaling pathways, and, as more recently reported, to mediate scavenger activity toward apoptotic cells. In addition, P2X7 exhibits a high number of single nucleotide polymorphisms (SNPs) and splice variants, several of which have been shown to impair ATP-mediated macropore formation. The aim of this study was to determine whether specific SNPs or deletion variants that have lost channel conductance or macropore activity retain other reported P2X7 functions. To address this, we analyzed the following variants: P2X7A (wild-type), P2X7B (unable to activate the macropore), P2X7 T283M (lacking conductance and macropore formation), P2X7 with N-terminal deletion (Δ N; deficient in signal transduction), and P2X7 DN (dominant-negative double mutant W167A/C168A, lacking all known activities). We evaluated calcium influx, macropore formation, ERK and p38 signaling, and scavenger activity. Our results indicate that macropore formation depends on channel conductance, in contrast to what has been previously reported for P2X7B. Moreover, all modifications tested impaired signaling pathway activation. Strikingly, none of the mutations affected receptor-mediated phagocytic activity. These findings suggest that loss of conductance or macropore formation does not necessarily entail loss of other P2X7 functions, and our data reveals a functional independence between scavenger activity and the canonical roles of P2X7 (channel/macropore, MAPKs). This modular view provides a framework to reconcile the apparently discordant phenotypes of P2X7 variants observed across diverse pathophysiological settings.



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1. Introduction

The ionotropic purinergic receptor P2X7 belongs to the P2X family but differs from other members due to its low affinity for ATP and its ability to form a macropore permeable to molecules up to ~900 Da when exposed to high, sustained ATP. Activation of P2X7 triggers distinctive cellular processes, including inflammasome activation in macrophages, which is crucial for interleukin-1 β production (priming and release of cytokine IL-1 β in microglial cells from the retina) [1]. This phenomenon can be elicited not only by ATP but also by proteins such as serum amyloid A [2], the β -amyloid peptide [3], and LL37, a cathelicidin-derived peptide [4].

Beyond ion conductance and macropore formation, P2X7 receptor participates in non-canonical functions that include activation of signaling pathways such as ERK, p38, Phospholipase D (PLD), and sphingomyelinase, largely independent of Ca²⁺ influx [5–8]. Additional activities described in the absence of extracellular ATP include (1) cell–cell fusion [9,10], (2) cross-dressing [11], and (3) a scavenger-type function that mediates uptake of apoptotic cells, bacteria, and non-opsonized particles [12,13]. A further hallmark of P2X7 is the abundance of single-nucleotide polymorphisms (SNPs) and splice variants. Many are associated with loss of canonical functions, particularly macropore formation. For example, the P2X7B variant and the homozygous E496A substitution yield receptors unable to open the macropore [14–16]. Likewise, mutations in the N-terminal region impair conductance and signaling, including ERK MAPK activation [5,17]. The T283M SNP produces a non-functional receptor lacking both conductance and macropore activity [18]. However, most studies of these variants have focused almost exclusively on canonical losses of function, with limited assessment of non-canonical activities.

The importance of these variants spans multiple pathophysiological contexts. The truncated isoform P2X7J, for instance, is expressed in highly metastatic cervical cancer, although its precise role in tumor progression remains unclear [19]. In inflammatory disease, such as graft-versus-host disease, P2X7 has been shown to be critical for immune activation [20,21], and certain SNPs have been linked to differential susceptibility to infections like pulmonary and extrapulmonary tuberculosis [22,23]. This functional and genetic diversity complicates a unified understanding of the receptor's roles in physiology and disease.

In this work, we sought to determine whether P2X7 variants previously characterized as “loss-of-function” for specific activities (calcium influx, macropore formation, or signaling) retain non-canonical functions, particularly scavenger activity. We analyzed P2X7A (wild-type), P2X7B (macropore-deficient), P2X7 T283M (lacking conductance and macropore), P2X7 Δ N (N-terminal deletion; signaling-deficient), and P2X7 DN (a double mutant with global loss of function), whose main features are summarized in Table 1. We comparatively evaluated macropore opening, conductance, ERK/p38 activation, and scavenger activity to test whether P2X7 functions can segregate and, consequently, whether specific variants might account for seemingly contradictory findings across different pathophysiological settings.

Table 1. Summary of the structural and functional characteristics of the main P2X7 variants analyzed in this study.

Variant/Mutation	Type of Modification	Functional Characteristics	References
P2X7A (WT)	None (full-length, canonical form)	Fully functional receptor with robust ionic conductance, macropore formation, ERK/p38 signaling activation, and non-canonical functions such as phagocytosis.	—
P2X7B	Splice variant lacking the C-terminal domain	Unable to open the macropore; reduced ATP affinity; may retain partial signaling or non-canonical functions.	[14–16]
P2X7 T283M (LOCHF)	SNP (T → M at position 283)	Non-functional receptor; lacks both ionic conductance and macropore activity.	[18]
P2X7 Δ N	Deletion of the N-terminal domain	Signaling-deficient (fails to activate ERK/MAPK pathways).	[5]
P2X7 DN (W167A, C168A)	Double point mutation (W167A + C168A)	No ionic channel and macropore; dominant-negative effect on other variants.	[24]

2. Results

2.1. Loss of BzATP-Induced Macropore Formation in P2X7 Variants

First, we evaluated whether the P2X7 receptor was expressed in the transfected cells. To this end, we performed Western blot analysis to detect P2X7 using two different antibodies. As shown in Supplementary Figure S1, only the APR-004 antibody detected P2X7 in the transfected cells, revealing a band close to 75 kDa. No signal was detected for the P2X7B variant, which was expected since the antibody epitope is located at the C-terminal region of the receptor and this variant lacks this region. Unfortunately, when the same samples were probed with the PA5-28020 antibody, bands were observed in all lanes, including the non-transfected cells, and these bands did not correspond to the expected molecular weight of P2X7.

Next, we assessed the macropore activity of the P2X7 receptor by flow cytometry, quantifying BzATP-induced ethidium bromide (EtBr) uptake. First, the response was characterized in HEK293 cells expressing the rat P2X7A isoform (WT). Stimulation with BzATP produced a dose-dependent curve at 10 min, with an apparent EC_{50} of $7.61 \pm 1.14 \mu\text{M}$, a Hill slope of 1.95 ± 0.38 , and a maximal response between 30 and 60 μM (Figure 1A,B); moreover, we found that the maximal EtBr uptake occurred at 20 min in P2X7A-expressing cells (Figure 1C,D). This response was specific to transfection, as it was not detected in the parental line used as a negative control.

Following this, we examined the macropore activity in several variants: P2X7B (a splice variant with reduced ATP affinity), P2X7 T283M (LOCHF, lacking ionic conductance and macropore activity), P2X7 W167A C168A (DN, a mutant with complete loss of function and dominant-negative effect), and P2X7 Δ N (N-terminal deletion, lacking transduction activity and showing low channel activity). Cells expressing these variants were stimulated with 60 μM BzATP for 20 min, and EtBr uptake was measured at 0, 15, and 20 min. The P2X7 LOCHF and P2X7 DN variants displayed a slightly macropore activation during the study period, whereas P2X7B and Δ N showed no activity. These results confirm previous reports for the P2X7B isoform and extend these observations to the other mutants, for which only channel activity loss had been described (Figure 1C,D).

In addition to macropore assessment, we measured the intracellular calcium influx in response to BzATP using flow cytometry. In P2X7A (WT)-transfected cells, BzATP stimulation induced a dose-dependent increase in intracellular calcium after 1 min (Figure 2A). In contrast, receptor variants lacking macropore activity did not exhibit calcium increases when tested at the maximal BzATP concentration used in the dose–response curve (60 μM), and no response was detected in the parental cells used as a control (Figure 2B).

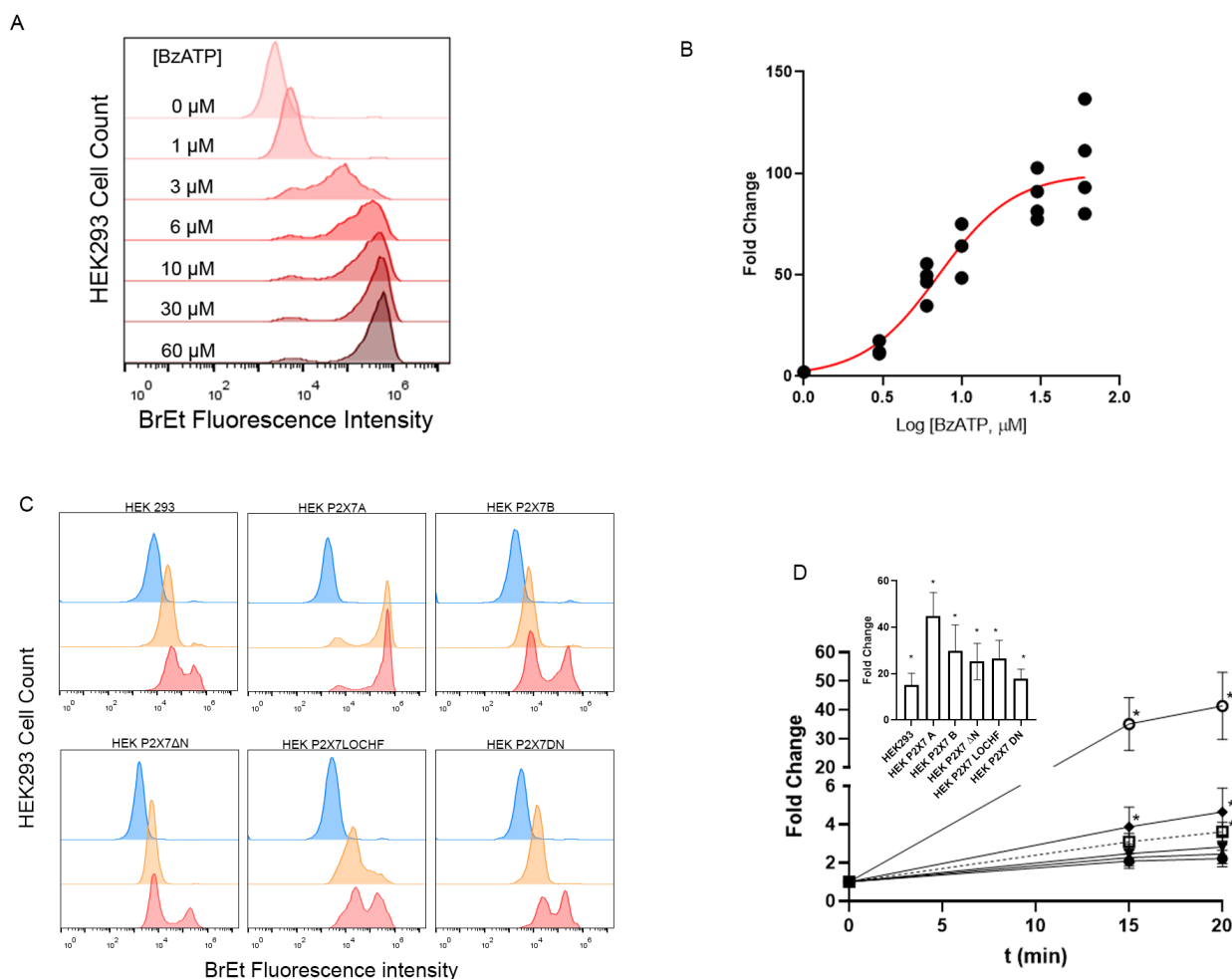


Figure 1. Rat P2X7A exhibits macropore activity. **(A)** Representative flow cytometry histograms showing ethidium bromide (EtBr) uptake in HEK293-P2X7A cells stimulated with increasing BzATP concentrations. **(B)** Dose–response curve of EtBr uptake versus log [BzATP], ($EC_{50} = 7.61 \pm 1.14 \mu M$, $n = 4$). **(C)** Representative histograms of EtBr uptake in HEK293 cells expressing P2X7 variants: basal (blue), 60 μM BzATP for 20 min (yellow), and Triton X-100 (red, positive control). **(D)** Fold change in FL2 fluorescence relative to baseline at 0, 15, and 20 min. Symbols: ● HEK293, ○ P2X7A, ▲ P2X7B, ▼ P2X7 Δ N, ◆ P2X7LOCHE, □ with dash line P2X7DN. Inset Triton X-100 positive control. Bars = mean $\pm$ S.E.M. of 6 experiments. * = $p < 0.05$.

2.2. Reduced MAPK Signaling in Cells Expressing P2X7 Variants

Our next objective was to evaluate whether the variants were capable of inducing signaling in response to BzATP, measured as MAPK activation. ERK activation was analyzed following stimulation with a fixed concentration of BzATP, determining its activation kinetics as previously described [25]. As shown in Figure 3A,B, BzATP induced ERK activation with a peak at 10 min in HEK-P2X7A cells, with phosphorylated levels remaining elevated until the end of the experiment. This response was absent in parental cells.

Next, we evaluate the ERK activation in the variants challenged with 60 μM BzATP at different time points. As shown in Figure 3A,B, the agonist did not induce significant ERK activation in the P2X7 Δ N mutant. Unexpectedly, P2X7 T238M displayed activation levels at the peak that were nearly comparable to P2X7A, but lacked the late-phase activation observed in the wild-type receptor. Meanwhile, P2X7B and P2X7 DN exhibited only partial activation at the peak with no sustained signaling.

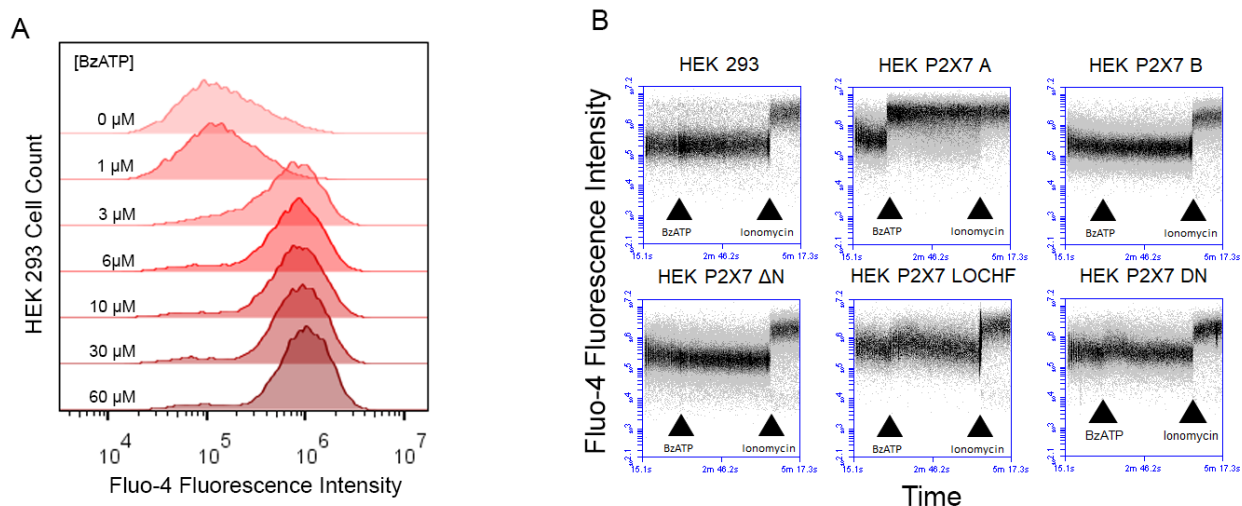


Figure 2. Rat P2X7A exhibits calcium influx. (A) Representative flow cytometry histograms of Fluo-4 fluorescence in HEK293-P2X7A cells stimulated with increasing BzATP concentrations. (B) Representative plots of P2X7 variants; black triangles indicate addition of 60 μ M BzATP (1 min) or 3 μ g/mL ionomycin positive control (4 min).

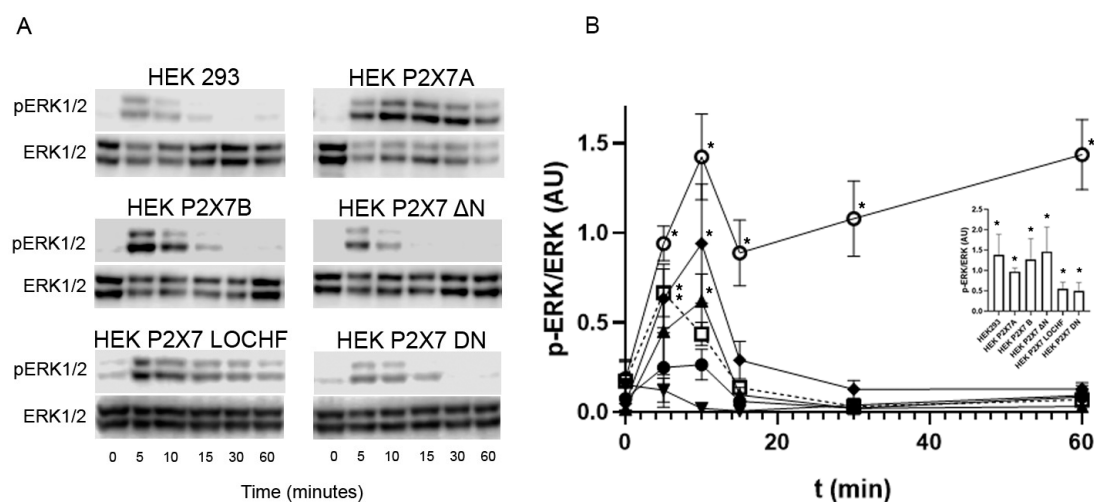


Figure 3. (A) Representative Western blots of phosphorylated and total ERK1/2 in HEK293 WT or rat P2X7 variant-transfected cells stimulated with 60 μ M BzATP. (B) Quantification of six experiments (pERK/total ERK, arbitrary units). Inset 40 nM PMA positive control. Symbols: ● HEK293, ○ P2X7A, ▲ P2X7B, ▼ P2X7 Δ N, ◆ P2X7LOCHF, □ with dash line P2X7DN. Bars = mean $\pm$ S.E.M. * = $p < 0.05$.

To determine whether similar effects occurred in other signaling pathways, p38 activation was assessed in the same samples tested for ERK. As shown in Figure 4A,B, BzATP induced a peak of p38 activation at 15 min in P2X7A, followed by a gradual decline up to 60 min. Among the variants, only the LOCHF mutant exhibited a comparable response to P2X7A at 5 min, maintained until 10 min but rapidly declining thereafter. These results demonstrate that MAPK signaling is largely dependent on the P2X7A isoform. The analyzed variants display partial, transient, or absent activation, confirming a differential functional loss in signal transduction.

2.3. A740003 Inhibits Macropore Activation but Not P2X7-Mediated ERK1/2 Signaling

Subsequently, we evaluated macropore activity and ERK pathway signaling in rat P2X7 wild-type, this time using the selective P2X7 inhibitor A740003. Figure 5A shows

that a 30 min pre-incubation with A740003 at 100 μ M inhibits EtBr uptake in HEK cells expressing wild-type P2X7 following stimulation with 60 μ M BzATP for 15 min.

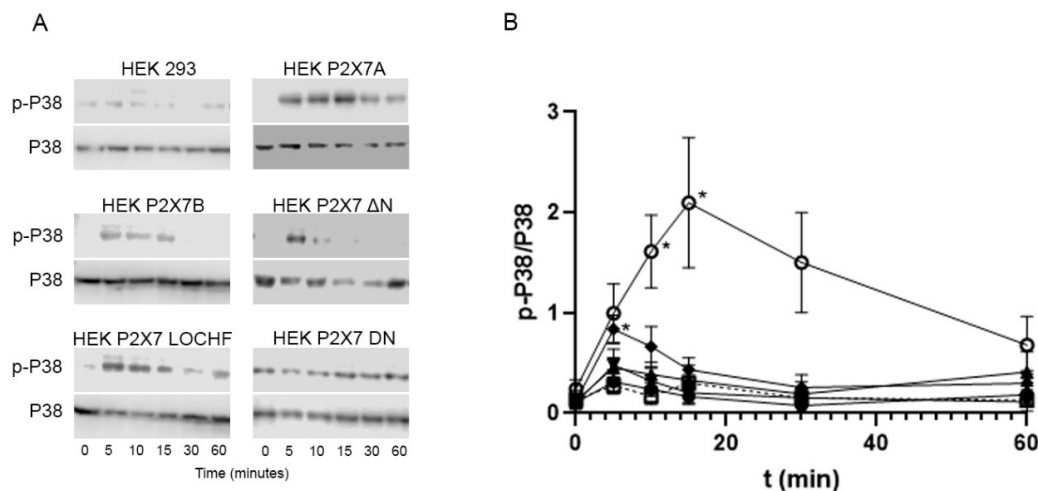


Figure 4. p38 activation in rat P2X7 variants. (A) Representative Western blots of phosphorylated and total p38 in HEK293 WT or P2X7 variant-transfected cells stimulated with 60 μ M BzATP. (B) Quantification of six experiments (p-p38/total p38, arbitrary units). Symbols: ● HEK293, ○ P2X7A, ▲ P2X7B, ▼ P2X7ΔN, ◆ P2X7LOCHF, □ with dash line P2X7DN. Bars = mean $\pm$ S.E.M. * = $p < 0.05$.

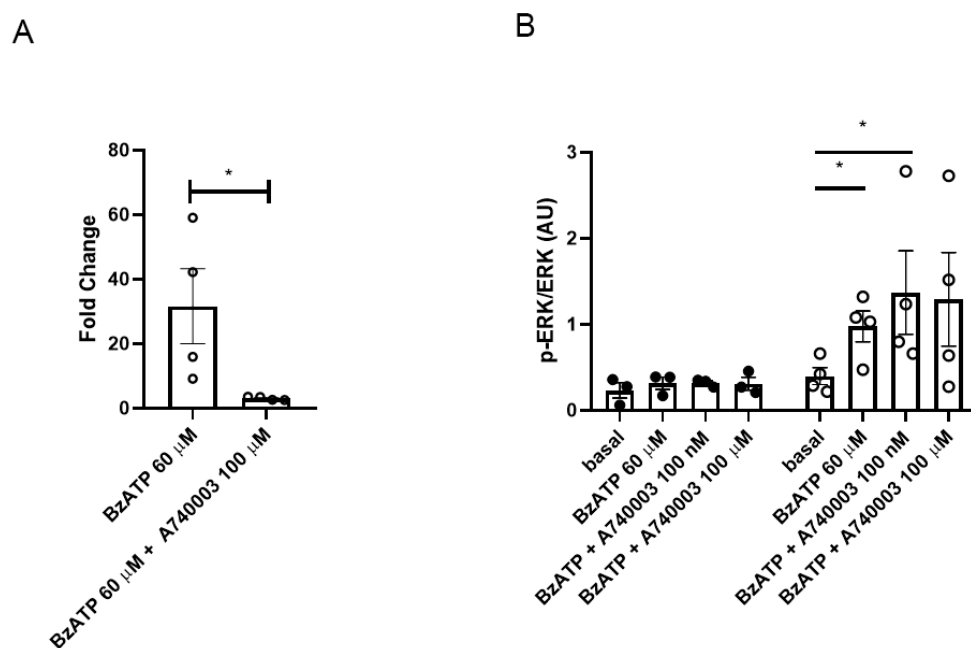


Figure 5. A740003 inhibits macropore activity of rat P2X7 but not ERK1/2 pathway signaling. (A) Quantification of ethidium bromide (EtBr) uptake in HEK P2X7A cells. (B) Quantification of ERK1/2 activation in HEK293 WT or HEK293 P2X7A cells. Symbols: ● HEK293, ○ P2X7A. Bars = mean $\pm$ S.E.M. of 4 experiments. * = $p < 0.05$.

In contrast to what was observed in the macropore-opening assay, analysis of the effect of A740003 on ERK1/2 activation revealed that the inhibitor had no impact. Figure 5B shows the quantification of phosphorylated ERK1/2 after a 30 min pre-incubation with A740003 at either 100 nM or 100 μ M, followed by stimulation with 60 μ M BzATP for 15 min in parental HEK293 cells or in cells expressing wild-type P2X7. No changes were detected in parental cells under any condition, whereas in P2X7-expressing cells, A740003 did not reverse the effect of BzATP on ERK1/2 activation.

2.4. Scavenger Activity Is Preserved Independent of the Loss of Macropore Function

Finally, the phagocytic activity of the receptor was evaluated following the method described by Gu et al. [26], in the absence of serum and using apoptotic bodies generated from MutuDC1940 dendritic cells endogenously expressing GFP. Apoptotic bodies were produced by nutrient deprivation, resulting in small sized structures as described previously by Barrera-Avalos et al. [11]. These apoptotic bodies were used to challenge HEK cells expressing DsRed-P2X7 or DsRed at a 1:1 cell-to-apoptotic body ratio for different incubation times.

As shown in Figure 6B, gating was performed on receptor- or DsRed-positive cells to evaluate phagocytosis. Cells transfected with DsRed alone did not show incorporation consistent with phagocytic activity, whereas P2X7A-expressing cells exhibited maximal uptake at 6 h of challenge, as demonstrated in representative histograms and experimental quantification.

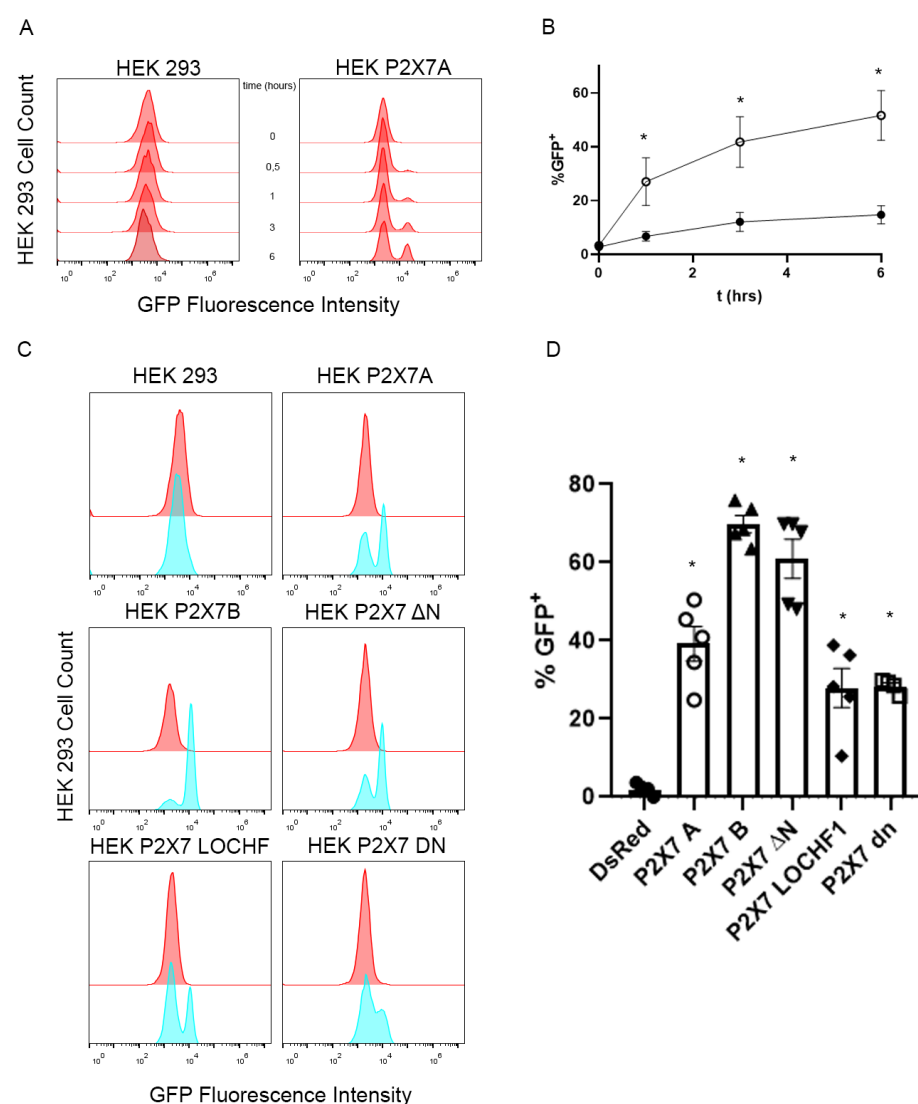


Figure 6. Phagocytic activity in rat P2X7 variants. (A) Representative flow cytometry histograms showing apoptotic body uptake between 0 and 6 h. (B) Quantification of phagocytic cells in HEK293 WT and P2X7A. Symbols: ● HEK293, ○ P2X7A. (C) Histograms of apoptotic body uptake in rat P2X7 variants at 0 h (red) and 6 h (blue). (D) Quantification of phagocytic cells per variant. Bars = mean ± S.E.M. of 4–6 experiments. Symbols: ● HEK293, ○ P2X7A, ▲ P2X7B, ▼ P2X7ΔN, ◆ P2X7LOCHF, □ P2X7DN. * = $p < 0.05$.

Once the optimal conditions for P2X7A-mediated phagocytosis were established, the variants were tested at 6 h using a 1:1 ratio with apoptotic bodies. Surprisingly, as shown in Figure 6C,D, phagocytic capacity was preserved in all variants, regardless of the specific functional loss. Remarkably, P2X7B and P2X7ΔN even displayed greater phagocytic activity than the wild-type receptor, reaching nearly 80% of cells with phagocytic capacity. These findings indicate that the phagocytic function of the P2X7 receptor remains intact across all tested variants. This suggests that phagocytic activity is independent of classical receptor functions such as macropore formation or ionic signaling.

3. Discussion

In this study, we assessed non-canonical functions of P2X7 across SNPs and mutants previously linked to loss of macropore formation, ion conductance, and signaling. Our data show that mutations abolishing “classical” functions (channel/macropore) differentially impair MAPK signaling (ERK/p38), while scavenger activity is preserved—and can even increase—in several variants. This functional dissociation supports the notion that P2X7 comprises partially independent activity modules, with implications for its pleiotropic roles in health and disease.

Compared with prior reports, we found notable discrepancies. In our study, P2X7 ΔN lacks Ca²⁺ influx and macropore opening, whereas Amstrup & Novak originally reported larger Ca²⁺ currents than P2X7A with BzATP. A plausible explanation is methodological: their N-terminal eGFP fusion may partially rescue the loss of residues 1–23. Subsequent work (e.g., Dreisig et al.) [17] showed N-terminal tags (eGFP/HA) disrupt channel/macropore, whereas C-terminal tags do not—consistent with our “tag-free” N-terminal deletion. Likewise, our P2X7B results align with the requirement for ~10-fold higher ATP to sustain any conductance; under standard conditions, this renders P2X7B functionally non-conductive. For DN and LOCHF mutants, we confirm non-activation of ion channel at 60 μM BzATP. In addition, we observed that macropore activity was reduced approximately tenfold compared to the full-length P2X7. These results indicate that P2X7 macropore activity is dependent on the ion channel function of the receptor.

Regarding MAPKs, variants exhibited distinct kinetic/amplitude profiles: P2X7A shows robust activation (ERK peak ~10 min; p38 peak ~15 min); ΔN fails to activate ERK and p38 substantially; P2X7B and DN display partial peaks without late phases; and LOCHF retains an early (ERK/p38) peak with rapid decay. These patterns support functional “pooling” of P2X7 dedicated to signaling vs. conductance (cf. García-Marcos et al.) [7] and indicate that early MAPK activation can occur partly independent of channel/macropore, at least in specific structural contexts.

Consistent with Gu et al. [12,13,26], we confirm that P2X7 confers phagocytic/scavenger capacity to HEK293 cells toward apoptotic bodies. Importantly, all tested variants retained this function; P2X7B and ΔN even showed enhanced phagocytosis (~80% positive), despite deficits in channel/macropore. Thus, scavenger activity is independent of ion conductance/macropore. The relationship between MAPK signaling and phagocytosis is intriguing: only LOCHF preserved early ERK1/2 and p38 peaks (rapidly decaying), whereas variants with higher phagocytosis lacked sustained signaling, consistent with segregated functional pools (signaling vs. scavenger). Mechanistically, such compartmentalization may rely on terminal domains, membrane microdomains, cytoskeletal coupling, and/or pannexin-1 complexes.

Additionally, in line with the concept that the canonical and non-canonical functions of P2X7 can be mechanistically decoupled, our results with the selective antagonist A740003 further support this notion. Although A740003 effectively inhibited macropore opening in cells expressing rat P2X7, it failed to inhibit P2X7-mediated ERK1/2 phosphorylation

induced by BzATP. This suggests that macropore formation and ERK pathway activation rely on distinct structural determinants within the receptor, and that blocking macropore activity is not sufficient to suppress kinase-mediated signaling.

Considering these findings, it becomes relevant to consider the structural and functional conservation of the P2X7 receptor across species, as this can provide insight into the extent to which its mechanisms may also be conserved. The rat and human P2X7 receptors are encoded by genes containing 13 exons and exhibit approximately 80% amino acid sequence identity, with the highest conservation observed in the extracellular and transmembrane domains. While the ATP-binding site is strongly conserved, the C-terminal region shows notable structural differences between species [27,28]. Moreover, several splice variants of the rat P2X7 gene have been described. Although these variants are not directly equivalent to those found in humans, functional comparisons indicate diminished receptor capabilities, largely derived from experimentally engineered mutations designed to probe specific aspects of P2X7 function.

Given the high degree of homology between the rat and human P2X7 receptors, the results obtained in this study may be extrapolated to the human receptor. However, due to the interspecies differences previously reported in sensitivity to pharmacological agonists and antagonists [29], it will be necessary to experimentally confirm whether these observations are maintained in the human receptor.

From a translational perspective, functional mapping of P2X7 isoforms/variants is critical. Isoforms deemed “afunctional” (e.g., P2X7J) may preserve scavenger-like regions with potential roles in cancer: hyper-stimulation of P2X7 can promote tumor cell death, yet LOF variants (e.g., P2X7B) may evade macropore-mediated apoptosis while partially maintaining ERK/p38 (proliferation, cytoskeletal remodeling, exosomes) and enhancing their phagocytic activity. Beyond cancer, GOF/LOF SNP combinations influence clinical outcomes (sepsis, neuropathic pain, infection susceptibility) and immune conditions (e.g., GVHD) with context-dependent and sometimes contradictory effects, emphasizing the need to disaggregate canonical vs. non-canonical activities to explain functional variability.

In this context, non-canonical P2X7 activities have shown relevance in pathophysiology. For instance, P2X7-mediated activation of p38 and Rho promotes exosome formation [30], which in turn may facilitate metastasis in malignant cells [31]. Moreover, certain human P2X7 variants are associated with increased susceptibility to infections or transplant rejection [20–22], potentially due to P2X7’s “cross-dressing” activity—where membrane patches containing MHC–antigen complexes are transferred from donor to antigen presenting cells—or its ability to activate the NLRP3 inflammasome. Variants with absent or enhanced versions of these non-canonical functions could therefore significantly influence these outcomes.

Overall, our data reveals a functional independence between scavenger activity and classical P2X7 functions (channel/macropore, MAPKs). This modular view offers a framework to reconcile the seemingly discordant phenotypes of P2X7 variants across diverse pathophysiological contexts.

Finally, the scope of our conclusions has certain limitations as, due to the poor quality of commercially available antibodies against P2X7, we were unable to reliably identify the presence of P2X7 variants at the plasma membrane using techniques such as flow cytometry or confocal microscopy. The nonspecific labeling obtained with these antibodies does not allow proper discrimination, as signal was also detected in non-transfected cells. Nevertheless, we were able to determine that the variants are expressed at similar levels by Western blot analysis. Taken together, and in line with our scavenger activity results, these findings indicate that the receptor is targeted to the plasma membrane, since all variants display this activity.

4. Methods

4.1. Cell Culture and Transfection

HEK 293 and MutuDC1940 cell lines were cultured under standard conditions. HEK 293 cells, kindly provided by Dr. Elias Leiva-Salcedo, were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), while MutuDC1940 cells, generously donated by Dr. Fabiola Osorio, were cultured in RPMI medium supplemented with 15% FBS and 50 μ M β -mercaptoethanol.

4.2. Plasmids

Plasmids purchased from GenScript (Piscataway, NJ, USA) were used. The corresponding cDNAs were cloned into pCDNA 3.1 hygro (+) vector containing the following rat genes: P2X7A, P2X7B, P2X7 T283M (P2X7 LOCHF), P2X7 W167A C168A (P2X7 DN), or P2X7 with N-terminal deletion (P2X7 Δ N).

4.3. Electroporation Protocol

HEK293 cells were resuspended in sterile PBS at a concentration of 2×10^6 cells/mL and mixed with the previously described plasmids at a final concentration of 10 μ g/mL. A 0.5 mL aliquot of this mixture was transferred to a Gene Pulser cuvette with 0.2 cm electrode gap (Bio-Rad, Hercules, CA, USA). Electroporation was performed using a Gene Pulser Xcell system (Bio-Rad) with the preset program for HEK 293 cells. Subsequently, transfected cells were maintained under selection with hygromycin at 100 μ g/mL, and receptor expression was characterized by Western blot analysis.

4.4. Apoptotic Body Preparation

Apoptotic bodies from MutuDC1940 cells were prepared by nutrient deprivation. The culture medium from a confluent cell plate was replaced with PBS and incubated at 37 °C for one week. Subsequently, apoptotic bodies were harvested, resuspended in 1 mL PBS, and quantified using a hemocytometer.

4.5. For Phagocytosis Assays

HEK 293 DsRed-P2A or HEK DsRed-P2A-rat P2X7 (WT or variants) cells (1×10^5) were seeded in 24-well plates and challenged with MutuDC1940 apoptotic bodies at a 1:1 ratio in the absence of fetal bovine serum (FBS). Cells were incubated at 37 °C, and a time-course analysis was initially performed with measurement points at 0, 0.5, 1, 3, and 6 h to assess phagocytosis by flow cytometry (BD Accuri C6). The percentage of HEK cells displaying dual fluorescence was measured: red fluorescence (from transfected DsRed expression) and green fluorescence (GFP from phagocytosed MutuDC1940 apoptotic bodies). Once the optimal phagocytosis time point was determined, the procedure was repeated using different P2X7 variants.

4.6. Macropore Activation

HEK 293 cells (1×10^6), either wild-type or transfected with rat P2X7, were resuspended in 1 mL PBS and labeled with ethidium bromide (EtBr, 25 μ M). This activity was assessed by flow cytometry measuring EtBr uptake induced by BzATP (Sigma-Aldrich, St. Louis, MO, USA). Cells were stimulated with different concentrations of BzATP (1–60 μ M), and fluorescence was recorded at 10 min. For kinetic assays, 60 μ M BzATP was applied, and EtBr uptake was measured at 0, 15, and 20 min. To assess the effect of the selective P2X7 inhibitor A740003, HEK293 P2X7A cells were pre-incubated with the inhibitor at 100 μ M for 30 min. The macropore opening was then evaluated by stimulating the cells with 60 μ M BzATP for 15 min, after which EtBr uptake was measured by flow cytometry.

4.7. Calcium Influx

HEK 293 cells (1×10^6), either wild-type or transfected with rat P2X7, were resuspended in 1 mL PBS and labeled with 2 μ M Fluo-4, AM (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After 30 min of incubation, cells were washed and resuspended in PBS supplemented with 1 mM CaCl_2 . For the dose-response assay, Ca^{2+} influx in the P2X7A variant was evaluated by flow cytometry, measuring fluorescence after 1 min of stimulation with BzATP at concentrations of 0, 1, 3, 6, 10, 30, and 60 μ M. To assess calcium activity in the other P2X7 variants, Fluo-4 fluorescence was monitored for 7 min using the BD Accuri C6 flow cytometer. The protocol consisted of 1 min of baseline fluorescence measurement, 3 min following BzATP 60 μ M addition, and a final 3 min after adding 3 μ g/mL ionomycin.

4.8. MAPK Activation

5×10^5 wild-type or rat P2X7-transfected HEK 293 cells were seeded in 6-well plates and incubated overnight. Prior to treatment, cells were serum-starved for 30 min to synchronize cells. Then, cells were subsequently treated with 60 μ M BzATP for variable time periods (0, 5, 10, 15, 30, and 60 min) or with 40 nM PMA for 30 min as a positive control for MAPK activation.

To assess the effect of the selective P2X7 inhibitor A740003, HEK293 or HEK P2X7A cells were pre-incubated with the inhibitor at 100 nM or 100 μ M for 30 min and were subsequently stimulated with 60 μ M BzATP for 15 min.

Following treatments, cells were immediately lysed with SDS-PAGE loading buffer supplemented with 5% β -mercaptoethanol and heated to 70 $^{\circ}\text{C}$. Cell lysates were homogenized by sonication using a Pulse 150 ultrasonic homogenizer (Benchmark Scientific, Sayreville, NJ, USA).

Protein samples were separated by electrophoresis on 10% acrylamide SDS-PAGE gels and transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad) with the preset program for high molecular weight proteins. Membranes were blocked with 4% BSA in TBS-Tween 20 buffer for 1 h at room temperature, followed by overnight incubation at 4 $^{\circ}\text{C}$ with the following primary antibodies diluted 1:1000: anti-phospho-ERK1/2 (Thr202/Tyr204) #9101S (Cell Signaling Technology, Danvers, MA, USA), anti-total ERK1/2 #4695S (Cell Signaling Technology), anti-total p38 #8690S (Cell Signaling), and anti-phospho-p38 (Thr180/Tyr182) #4511S (Cell Signaling Technology). Subsequently, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody #1721019 (Bio-Rad) diluted 1:2000 for 1 h at room temperature. Detection was performed using Pierce ECL Western blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and images were captured using the C-DIGiT Model 3600 system (LI-COR, Lincoln, NE, USA).

4.9. Analysis of P2X7 Expression

To evaluate P2X7 expression, pooled samples from the MAPK activation assay at time 0 for each variant were used. Samples were separated by electrophoresis and subjected to Western blot analysis under the conditions previously described. Anti-P2X7 antibodies APR-004 (Alomone Labs, Jerusalem, Israel), diluted 1:500, and PA5-28020 (Thermo Fisher Scientific), diluted 1:1000, were used. The secondary antibody incubation and signal detection were performed under the same conditions as described previously.

4.10. Statistical Analysis

EtBr uptake and ERK/p38 activation were evaluated by the Kruskal–Wallis ANOVA. Calcium influx and phagocytosis kinetics were evaluated with Mann–Whitney U-test. The differences in the percentage of phagocytosis between P2X7R variants were analyzed by

Wilcoxon Test. All the analyses were performed using the GraphPad Prism 8.01 software (GraphPad, San Diego, CA, USA). Results are presented as mean $\pm$ SEM, and statistical differences were considered significant at $p < 0.05$.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041922/s1>.

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