Molecular Pharmacology Fast Forward. Published on February 8, 2019 as DOI: 10.1124/mol.118.115022
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A mechanism based approach to P2X7 receptor action

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Running title: **P2X7 Receptor; beyond the pore formation.**

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Number of pages: 29

Number of boxes: 1

Number of figures: 1

Number of references: 72

Number of words in the abstract: 250

Number of words in the main text: 6236

Abbreviations: **ATP**: Adenosine triphosphate; **NMDG**: N_Methyl-D-glucamine; **P2X2(4,7)R**: P2X purinergic receptor 2(4,7); **TRPV**: transient receptor potential cation channel V; **YoPro-1**: 4-((3-methyl-2(3H)-benzoxazolylidene)methyl)-1-(3-(trimethylammonio)propyl)quinolinium diiodide; **ToTo-1**: 1,1'-(4,4,7,7-Tetramethyl-4,7-diazaundecamethylene)-bis-4-(3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)-quinolinium tetraiodide; **LDH**: Lactate dehydrogenase enzyme; **HEK293:** Human embryonic kidney cells; **PLD**: Phospho lipase D enzyme; **PLA2** Phospho lipase A2 enzyme; **PI4K**; 1-phosphatidylinositol 4-kinase enzyme; **PI3K**: Phosphatidylinositol-4,5-bisphosphate 3-kinase; **EMP-1(2,3)**: Epithelial membrane protein 1(2,3); **Ni-AchR**: Nicotinic acethylcholine receptor. **AKT**: Protein Kinase B; **NF-κB**: nuclear factor kappa B. **GSK3β**: Glycogen synthase kinase 3 beta. **IL-1β:** Interleukin 1 beta.

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Abstract:

Ligand-gated ion channel P2X7 receptor attracts special attention due to its widespread presence as well as its unusual responses. Besides relatively well understood mechanisms such as intracellular Ca^{2+} increase and K^{+} depletion, P2X7 receptor activates other peculiar responses whose mechanisms are not fully understood. The best known among these is the permeabilization of the cell membrane to large molecules. This permeabilization has been explained by the activation of a non-selective permeation pathway by the P2X7 receptor, a phenomenon called “pore formation”. However, with the emergence of new data, it became apparent that large molecules enter the cell directly through the pore of the ion channel similar to the smaller ions. This explanation seems to be true for cationic large molecules. On the other hand, there is still convincing evidence indicating that P2X7 receptor activates a separate pathway which permeates anionic large molecules in some cell types. Furthermore, there exists functional data suggesting that P2X7 receptor may also activate other intracellular signaling molecules or other ion channels. Interestingly and contrary to what is expected from a ligand-gated channel, these activations occur in a seemingly direct manner. Somewhat overshadowed by the “pore formation” hypothesis, these action mechanisms may lead to a better understanding of not only the P2X7 receptor itself but also some important physiological functions such as the release of anionic autocoids/neurotransmitters in the central nervous system. This review aims at discussing, assessing and drawing attention to the data concerning these neglected but potentially important points in the P2X7 receptor field.

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Introduction:

P2X7 receptor (P2X7R) belongs to the P2X receptor family, all seven members (P2X1-7) of which are widely accepted as ligand-gated ion channels that can be activated by extracellular ATP. However, this receptor is known to have other peculiar actions that distinguish it from other ligand-gated channels. Along with molecular interventions including knock-out of the receptor, vast amount of experimental evidence based on pharmacological experiments with specific agonists and antagonists show that P2X7R is involved in a diverse set of cellular responses.

Recent interest in P2X7R field concentrates mostly around areas of cancer and inflammation. ATP has been seen as an important mediator in inflammation and tumor biology as its concentration can reach high levels around the cells in an inflamed area or inside a tumor, thus potentially providing a sustained background stimulus regulating and affecting the function of immune and neoplastic cells. With its relatively low sensitivity to ATP, P2X7R is a good candidate for a receptor that will function in such a high-ATP environment. Concordantly, several studies suggest that P2X7R is an important, even a central player in key processes related to inflammation and cancer such as the activation of IL-1β processing most probably via NLRP3 inflammasome complex in macrophage-like immune cells (Ferrari et al. 1997a; Solle et al. 2001; for details see Adinolfi et al. 2018), in vivo growth (Adinolfi et al. 2012; Amoroso et al. 2015) and invasion of cancer cells (Xia et al. 2015; Ferrari et al. 2006; Di Virgilio et al. 2017; Di Virgilio and Adinolfi 2017). As we shall see throughout this review, involvement of P2X7R in physiology and pathophysiology is actually much wider than these examples suggest. On the other hand, despite the vast amount of effort put in the field, the mechanism(s) of action for P2X7R is still not-well understood. Therefore, this review's main focus will be the mechanisms of action that P2X7R uses to activate cellular responses rather than being what those responses are or what pathological processes this receptor is involved in. We will use examples of physiological/pathological phenomena where P2X7R is thought to play important roles only with the intention to illustrate our points and to give an idea of the implications of the mechanisms that we discuss in the review. For a general view of the structure, pharmacology or the wide range of physiological and pathological actions of P2X7R, following comprehensive reviews are recommended (Di Virgilio and Adinolfi 2017; Di Virgilio et al. 2017; Di Virgilio et al. 2018; Peverini et al. 2018; Young and Gorecki 2018; Adinolfi et al. 2018).

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Since the initial observations made in mast and transformed 3T3 cells (Rozengurt et al. 1977; Cockcroft and Gomperts 1980) and later in macrophages (Steinberg et al. 1987), ATP has been known to induce uptake of both the cationic and the anionic large organic molecules such as fluorescent dyes into the cells as well as release of some metabolites (mostly anionic) from the cell. The hypothetical ATP receptor which mediates this permeabilization response was initially called P2Z. Later, the cloned ligand-gated channel protein P2X7R was identified to be this ATP receptor (Surprenant et al. 1996). In due course, it was postulated that P2X7R permeabilized cells to large molecules either by dilation of its own pore (Surprenant et al. 1996) or activation of another non-selective large pore forming protein (Pelegrin and Surprenant 2006). This mechanism was named as "large pore formation" (or simply "pore formation") and P2X7R was thought to be unique in this regard. For a long time, "large pore formation" had been the central mechanism to explain P2X7R actions. However, P2X7R neither appears to be unique in increasing membrane permeability to large molecules nor does it seem to activate large pores as will be discussed below in detail. In the light of recent experimental findings, the P2X7R-induced permeability to large molecules is now thought to be simply a direct result of the opening of the P2X7R cation-channel.

In our belief, neither the debate on permeation to large molecules nor the issues regarding other unique interactions of P2X7R has reached a satisfactory conclusion. Moreover, the over-emphasis on "pore formation" mechanism obscured the data which may hold important clues for the probable mechanisms of P2X7R action that are much more diverse than what was thought before. This review aims at presenting and discussing these mechanisms in a clear way. We will try to do this by forming testable working-hypotheses based on a comprehensive review and a detailed discussion of the available data. Although we will discuss all the aspects of the P2X7R action mechanisms, we believe that two mechanisms not prominently covered in the literature need special attention: The first one is a less-appreciated mechanism for large molecule permeation. The second is the possible direct interaction of the P2X7R with intracellular signaling pathways.

In general, three not mutually exclusive mechanisms of action are proposed for the P2X7R:

1) As a regular ligand-gated ion channel, the receptor, upon stimulation, may increase the cell membrane permeability to small cations leading to depolarization of the cell membrane, Ca^{2+} uptake into the cell or efflux of K^+ from the cell.

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2) It may increase the permeability of the cell membrane to large molecules that are too large to go through regular ion channels, leading to either uptake or loss of big molecules such as nucleotides. As mentioned above, the so called “pore formation” or “cytolytic action”, has been the most popular mechanism to explain this permeability. It was proposed to happen either through a gradual dilation of the ion channel pore or activation of an accessory protein which can conduct large molecules.

3) Through direct interaction, it may activate some intracellular signal transduction proteins and cellular processes.

Mechanism 1: Is P2X7R a Ligand-Gated Cation-Channel?

There is little doubt that P2X7R is a ligand-gated cation-channel. However, whether or not it is a regular cation-channel similar toNi-AchR is quite controversial. Because, in the course of stimulation, it seems to permeate large cationic and also anionic molecules which normally should not go through ion channels. The question as to whether P2X7R ion channel goes through a “pore dilation” process for this permeation to occur will be discussed below. Nevertheless, as P2X7R activation is known to cause a permeability increase to anionic molecules as well, the first question to ask is whether the P2X7R really forms a cation-selective conduit or is a non-selective channel (i.e. permeates both cations and anions). Interestingly, the results of the electrophysiological experiments concerning this question fall into two categories according to the experimental system used. The first category contains the results obtained in **heterologous expression systems.** In electrophysiological experiments conducted in expression systems which do not express the receptor endogenously, P2X7R activation mostly leads to a cation-selective whole-cell current (Virginio et al. 1999a) and a cation-selective single channel activity, with no significant selectivity between small cations such as Na^+, K^+ or Ca^{2+} (Riedel et al 2007 a and b). P2X7R permeation ratio for Cl^-:Na^+ was reported to be about 1:100 or less. Thus, compared to cations this ionic current/channel is almost impermeable to anions, including Cl^-. (Virginio et al. 1999a; Kubick et al. 2011; Browne et al. 2013). P2X7R current has been shown to be carried not only by Na^+, K^+, Ca^{2+} but also by large organic cations such as $NMDG^+$ or $Tris^+$, the largest of those being $Tetraetylamonium^+$ (130 Da) (Riedel et al 2007 a and b). However, as expected, the large cations have smaller permeabilities. The second category comprises the results obtained in **cells that naturally express the P2X7R such as mast cells and astrocytes.** Examples in this category are intriguing, but unfortunately few. In these cells, ATP stimulation seems to activate

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a non selective current, conducting both anions (including Cl^-, glutamate$^-$ and aspartate$^-$) and cations in the whole-cell mode (Tatham and Lindau 1990; Duan et al. 2003).

As mentioned above, the other important question concerning the P2X7R is whether its activation gradually increases the permeability of the membrane through either enlargement of receptor's own ionic pore or the activation of another "pore" protein to allow the passage of molecules that are usually too large to pass through the ion channels. In other words, does "pore formation" occur? Pore formation theory was based on the initial electrophysiological observation that prolonged activation of P2X7R led to a time-dependent change in reversal potential of the whole-cell currents; a gradual shift from more negative to significantly less negative values (Surprenant et al. 1996). Assessment of reversal potentials of ionic currents is the usual method to determine relative permeability of ion channels to different ions. These measurements are conducted under conditions where the ions such as Na^+ or K^+, are replaced with large organic molecules with a similar charge ($NMDG^+$ or $Tris^+$). This so called "ion replacement" is done asymmetrically: i.e., usually only in the bathing solution. According to the Goldman-Hodgkin-Katz equation, two factors act together to determine the reversal potential of the membrane current; the relative permeabilities of the ions and the ratio of their extra to intra cellular concentrations (i.e. Nerst potential of the ion). For the regular ion channels, the reversal potential of the current is expected to be a negative value under the asymmetric ionic replacement conditions described above as the large ions such as $NMDG^+$ has no significant permeation and thus the current is mostly determined by the small ions (Na+ or K+) which have negative Nerst potentials. As expected, a negative reversal potential was observed for the whole cell current at the initial phase of the P2X7R activation. Unexpectedly, however, the reversal potential gradually changed from negative values towards zero. This well-repeated observation was interpreted as an indication of P2X7R channel becoming more permeable to large organic cations (i.e. opening of a large **non selective pore). Let us reiterate that this proposed "large non selective pore" opening does not mean that the P2X7R current becomes non-selective between cations and anions**; the current seems to stay cation-selective and does not permeate anions. It is thought to loose its selectivity between large and small cations and gradually become more permeable to **large cations** (Virginio et al. 1999a). Let us also state that this gradual shift in the reversal potential was later found not to be unique for P2X7R and described for other ligand-gated ion channels such as P2X2R, P2X4R (Virginio et al. 1999b; Khakh et al. 1999) and TRPV1 (Chung et al. 2008).

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In the case of P2X7R, enlargement of the receptor's pore itself was proposed to explain this permeability increase to large cations. However, more recent single channel experiments gave results quite inconsistent with this ion channel-to-pore enlargement idea. Single channel data suggest that, in contrast to the whole cell currents, P2X7R single channel currents have a very stable reversal potential as expected from a regular ion channel. Nevertheless, the P2X7R channel is somewhat more permeable to large cations than many other ion channels are. This is the case from the beginning of its activation; its permeability characteristics do not change in time (Riedel et al. 2007 a and b, see also Harkat et al. (2017) for a similar finding in P2X2R). Furthermore, data obtained by cysteine accessibility mutations suggest that P2X7R conduction pore does not show any sign of dilation during receptor activation (Pippel et al. 2017).

Even if the single channel data indicate that P2X7R pore itself does not enlarge, it is still possible that an additional conduction pathway which is gradually activated by the receptor in the whole-cell settings may increase the permeability to large cations. However, a recent study elegantly and convincingly demonstrates that this is also not the case and the reversal shift observed in the whole cell setting is likely to be an artifact (Li et al. 2015). In the ion replacement experiments where the reversal potential shift was observed, the ionic concentrations of the intra- and extra-cellular solutions were assumed to be stable throughout the experiment. Hence, the observed reversal potential change was attributed to an increase in the permeability to large ions. However, the study by Li et al. (2015) shows that this assumption is not justified in the case of P2X2R: Intracellular concentration of the large cations used in the extracellular solution for the ion replacement is gradually increased due to the large and persistent P2X2R-activated membrane conductance. This increase is the result of the small but measurable permeation of these large cations through the P2X2R channel. Additionally, intracellular small ion concentration decreases during P2X2R activation. Thus, after the findings of Li et al. (2015), the reversal potential shift can very well be explained by the change in the composition of the intra-cellular solution rather than an increased permeability to large cations. Even though the observation was made for P2X2R, the main argument is relevant for all the receptors assumed to exhibit "pore dilation", including P2X7R. Hence, for P2X7R, there seems to be no "pore dilation" or "pore activation" but simply opening of a cation-channel which is somewhat permeable to large cations. A more detailed discussion of the subject can be found in Peverini et al. (2018). Additionally, the idea that proteins such as pannexins, activated by P2X7R form a pathway that permeates large cationic molecules

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(Pelegrin and Surprenant 2006) were not confirmed by later studies (e.g. Qu et al. 2011; Hanley et al. 2012; Alberto et al. 2013).

Thus, the experimental results discussed in this section lead to three general conclusions:

i) When heterologously expressed, P2X7R forms a ligand-gated ion channel which is strictly cation-selective and besides small cations, permeates larger organic cations such as Tetramethylamonium.

ii) The selectivity of the P2X7R-activated current does not change with time and terms like "pore dilation", "channel to pore transition" or "pore activation" are not correct descriptions of what actually happens.

iii) Despite the facts in (i) and (ii), P2X7R is able to induce a non-selective whole-cell current which permeates both cations and anions in certain cell types that naturally express the receptor.

Ligand-gated ion channel activity is the best established mechanism for P2X7R. Being a cation-channel, P2X7R can cause Ca^{+2} influx and increase intracellular Ca^{+2} concentrations, as shown by numerous studies. Intracellular Ca^{+2}, increase was clearly shown to be the mechanism responsible for a number of P2X7R-induced responses, as in the case of AKT activation in astrocytes (Jacques-Silva et al. 2004). In other cases, however, involvement of the P2X7R's ion channel activity is not so clear-cut. For example, as mentioned above, NLRP3 complex-related IL-1β processing, an apparently crucial step in inflammation is triggered by P2X7R. K^+ efflux (more precisely the decrease of intracellular K^+ concentration due to this efflux), had been proposed to be the trigger for this response (Walev et al. 1995; Ferrari et al. 1997a). As it readily permeates K^+ ions and does not inactivate easily, P2X7R is a fairly suitable conduit for this role and the K^+ depletion was initially thought to occur through the P2X7R channel (for details, see Di Virgilio et al. 2017; Adinolfi et al. 2018). However, a recent study suggests that besides P2X7R, another ion channel, namely the TWIK2 K^+ channel is also essential for NLRP3 complex-related IL-1β processing (Di et al. 2018). This study showed that in macrophages, TWIK2 channel could clearly add a substantial K^+ conductivity to what could be provided by P2X7R alone. TWIK2-provided K^+ current seems to be essential for P2X7R-induced IL-1β processing, as this response is absent in TWIK2 knock-out $Kcnk6^{-/-}$ macrophages or in macrophages where TWIK2 is knocked down by using siRNA. Interestingly, however, this channel appears to be

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activated not by P2X7R but by another, yet unidentified, ATP receptor as TWIK2 currents can readily be activated by ATP in P2X7R$^{-/-}$ macrophages (Di et al. 2018). If K^+ efflux (intracellular K^+ depletion) and P2X7R activation are both essential for ATP-induced IL-1β processing as suggested by various studies, then according to the results of Di et al. (2018), it seems that the role of P2X7R in IL-1β processing is not increasing the K^+ conductance. P2X7R must then serve some other essential, yet unknown function. As Di et al. (2018) pointed out, this function can be to provide a Na^+ conductance to counter the hyperpolarizing effect of the increased K^+ conductance as hyperpolarization paradoxically diminishes the electro-chemical potential driving the K^+ efflux. As can be seen from this discussion, the action mechanism of P2X7R in IL-1β processing is still not fully understood and further investigation is needed.

Mechanism 2. Does P2X7R Permeabilize the Cells to Large Molecules?

As discussed above, the answer to this question is yes. Uptake of fluorescent dyes into cells is the most preferred tool to assess the permeability of cells to large molecules and P2X7R stimulation is well known to cause cell membranes to become permeable to fluorescent dyes. These dyes are considerably larger than the cationic molecules such as $NMDG^+$ that are used in electrophysiological studies. Before proceeding further, one point must be clarified here: With long P2X7R stimulations lasting for a few hours, cell death and other drastic and complex processes may permeabilize the cells to molecules as large as LDH (e.g. Ferrari et al. 1997b; Le Stunff and Raymond 2007). The P2X7R-induced permeabilization process that we are referring to should be distinguished from this type of permeabilization. What we are interested in here is a permeabilization that starts almost immediately after the P2X7R stimulation. We will be discussing observations lasting in the range of 10-20 minutes rather than hours and regarding the phenomenon that renders the plasma membrane permeable to molecules not larger than 1000 Da. Thus, the “large molecules” in question here are considerably small compared to LDH (MW ~140 000 Da). This said, we can now focus on an interesting property of the P2X7R-induced cell permeabilization. Similar to the ionic currents, the selectivity of the dye-uptake phenomenon also seems to be dependent on the experimental system used: P2X7R-stimulated uptake seems to be selective for cationic-dyes in heterologous expression systems, but tends to be non-selective when P2X7R is endogenously expressed in the cell.

Numerous observations clearly show that when a functioning P2X7R is externally expressed, a cationic dye (such as ethidium bromide or YoPro-1) uptake occurs (Surprenant et al.1996; Virginio et al. 1999a and many others). In contrast, the number of the studies which systematically investigated the permeation of anionic dyes by the activation of heterologously expressed P2X7R is limited. Nevertheless, two studies conducted in HEK293 cells expressing a transfected P2X7R, despite using very different experimental desgns, show clearly that P2X7R activation does not cause any anionic dye uptake. In both studies, while cationic-dyes as large as ToTo-1 (894 Da) could easily pass through, anionic ones such as lucifer yellow (433 Da), fluorescein (332 Da) or calcein (623 Da) were clearly excluded from the P2X7R-activated permeation pathway (Schachter et al. 2008; Cankurtaran et al. 2009). Although a Ca^{2+}-activated Lucifer Yellow uptake was also observable, this was not a P2X7R-dependent uptake as it could also be activated by a Calcium ionophore in parent HEK293 cells and it was possibly due to a Ca^{2+}-activated anion-channel (Cankurtaran et al. 2009).

This brings us to the question as to whether these large cationic-dyes go through the P2X7R channel itself as the small cations do, or through another permeation pathway. Cationic-dye uptake and cation-channel activity has been claimed to be dissociated by performing mutations or deletions (especially at the carboxy terminal) on the P2X7R receptor or by expressing it in oocytes. Thus, at first sight, separate permeation pathways seem to exist for small cations and large cationic-dyes. However, there are problems associated with this body of data, which can be summarized as follows: Deletion of a cysteine rich region in the carboxyl-terminal of the protein which was shown to reduce the ionic currents and the YoPro-1 uptake induced by P2X7R (Surprenant et al. 1996), later claimed to abolish the reversal potential shift but not the YoPro-1 uptake (Jiang et al. 2005). Contrary to this finding, Yan et al. (2008) found that the same deletion did not affect the reversal potential shift observed with P2X7R currents. A similar but naturally occurring truncation was also reported to abolish only the YoPro-1 uptake but not the ionic-currents (Adinolfi et al. 2010). Yet, on a closer look, what this mutation seems to do in the study by Adinolfi et al. (2010) is simply to reduce the small (Ca2+) and large (YoPro-1) cation uptake in a comparable way and in conjunction with the results of a previous study performed with another carboxyl-terminal truncated P2X7R (Adinolfi et al. 2005) it looks as if this truncation reduces the overall activity of the receptor. Such a loss in P2X7R activity with carboxyl tail deletion is also suggested by the findings of Karasawa et al. (2017). In another study, carboxyl-terminal truncation of P2X7R has been claimed not to affect the ionic currents but abolish the dye-

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uptake (Smart et al. 2003). However, the use of different cell types to measure ionic currents (oocytes) and the dye uptake (HEK-293) seems to be a major draw back of this study as magnitude of the functional loss caused by the truncation was later shown to change in a complicated way depending on the membrane lipid composition or the cell type (Karasawa et al. 2017). A similar argument can also be made for the effect of E496A mutation on P2X7R: This mutation was claimed to affect the dye uptake and ionic currents differentially as it abolished the cationic-dye uptake in one study (Gu et al. 2001) but did not affect the ionic currents in another one (Boldt et al. 2003). However, only a single type of response was measured in each study and in neither of them the state of the other response was checked. A loss of function due to carboxyl terminal truncation (or other alterations), affecting the large and the small cation permeation in a similar way is expected to reduce the dye uptake and, according to Li et al. (2015), also diminish the reversal potential shift. It appears that such a loss of function may explain the discrepant results given above for the truncated P2X7R, especially if this function loss is cell type-dependent as suggested by Karasawa et al. (2017). Still, why the reversal potential shift and dye uptake was affected in such a different way in Jiang et al. (2005) can not be explained easily by this argument.

Box1- Working hypotheses about action mechanisms of P2X7R :

1) P2X7R is a ligand-gated cation-channel that permeates large cationic molecules as big as the dye Yo-Pro but not anions such as lucifer yellow or even Cl^-. In this sense it is similar to other ligand-gated channels such as P2X2R and TRPs.

2) P2X7R activates an accessory pathway which permeates anionic large molecules. This unidentified permeation pathway exists only in some cell types and can be an anion transporter or an ion channel (Figure 1, first model). Its activation does not involve mechanisms related to the cation-channel activity of the receptor such as Ca^{2+} influx, K^+ depletion or depolarization.

3) P2X7R, in addition to its ligand-gated ion channel activity, can also interact directly with intracellular signaling pathways and activate other ion channels, enzymes and the anionic permeation pathway mentioned in the second hypothesis.

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The lack of reversal potential shift with P2X7R activated ionic currents when the protein is expressed in oocytes was also interpreted to indicate that P2X7R induced large and small ion permeability through separate pathways (Petrou et al. 1997). However when the unusually short duration of the inactivating P2X7R current they presented and the large volume of the oocyte are taken into consideration, the model proposed by Li et al. (2008) exactly predicts the lack of the reversal potential shift in the study of Petrou et al. (1997).

On the other hand, there exists an alternative group of data suggesting that large and small cations go through the same pore. When purified and reconstituted into lipid vesicles, carboxyl terminal truncated P2X7R, by itself, was sufficient for the uptake of the cationic dye YoPro-1, as well as Ca^{2+} and changing the lipid composition of the reconstitution vesicles, especially the cholesterol content, affected YoPro-1 and Ca^{2+} permeations in a similar way (Karasawa et al. 2017). This direct finding strongly supports the hypothesis that at least the cationic-dyes can permeate through the P2X7R channel. In another interesting study, where the dye uptake and the ionic-currents were measured simultaneously in voltage-clamped single cells, it was shown that the cationic dye uptake was in "lockstep" with the P2X7R-activated ionic-current in terms of both the time course and the current (flux)/voltage characteristics. Additionally, when the cation selectivity of the channel was altered by mutations on the P2X7R protein pore region, permeability to both Cl^- and negative dyes increased together, suggesting that small and large ions (dyes) go through the same pore (Browne et al. 2013). In conclusion, the experimental evidence supporting the hypothesis that the **cationic-dye uptake is through the P2X7R channel itself,** compared to those in favor of separate permeation pathways, seems to be more direct and internally coherent which bias us towards the idea that a single permeation pathway exists for all cations (see also Peverini et al. 2018). Here, it should be mentioned again that P2X7R is not unique in being permeable to large cationic-dyes and other ligand-gated cationic-channels such as P2X2, P2X4 (Virginio et al. 1999b; Khakh et al. 1999), TRPV1 (Chung et al. 2008) and TRPA1 (Banke et al. 2010) can induce the uptake of large cationic-dyes into cells.

Compared to the expression systems, the cells that naturally express the P2X7R exhibit a very different picture in terms of dye permeability. It is well known that ATP/BzATP application to macrophages, astrocytes or macrophage-derived cell lines such as J744 or RAW 264 causes permeability not only to cationic-dyes like YoPro-1 (Pelegrin and Surprenant 2006; Le Stunff and Raymond 2007; Schachter et al. 2008; Cankurtaran et al. 2009) but also to anionic-dyes such as

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Lucifer yellow (433 Da), fluorescein (332 Da), calcein (623 Da) (Steinberg et al. 1987; Duan et al. 2003; Fellin et al. 2006; Schachter et al. 2008; Cankurtaran et al. 2009), indo-1 (650 Da) (Greenberg et al. 1988) and fura-2 (831 Da) (Steinberg et al. 1987).This anionic-dye permebility is reversible as it ceases when extracellular ATP is washed away (Steinberg et al. 1987; Schachter et al. 2008; Cankurtaran et al. 2009). It is definitely not through pinocytosis (Steinberg et al. 1987) or not due to intracellular Ca^{2+} increase or K^{+} loss from the cells (Cankurtaran et al. 2009). Hence, if the ATP receptor in these cells is indeed P2X7R, as agonist and antagonist specificity data suggests, then the anionic dye uptake by P2X7R stimulation seems to be cell-type dependent (occurs in macrophages but not in HEK293 cells, for example). There is one other study with anecdotal evidence that should be mentioned here: In neuron-derived GT1 cells, stimulation of the externally expressed P2X7R seems to cause a clear leakage of pre loaded anionic-dyes fura-2 and fura-TT out of the cell, as well as a cationic dye uptake (Yan et al. 2008). This finding further suggests the cell-type dependence of the anionic-dye permeation.

Thus, together with the electrophysiological results presented in the previous section we may conclude that:

i) **Externally transfected P2X7R** activates **a cation-selective ionic current** and also **a cation-selective dye-uptake**.

ii) P2X7R ion channel seems to permeate large cations like Tetraetylamonium^{+} as well as larger cationic-dyes lie YoPro-1.

iii) In **cells which naturally express the P2X7R,** the receptor activates **a non selective current and a non selective dye-uptake, through which** both **anionic and cationic molecules can pass**.

iv) Additionally, as discussed in the previous section, evidence for the P2X7R-induced non-selective large molecule uptake mechanisms such as the pore dilation or the involvement of accessory pore proteins is not very strong.

The simplest model to encompass all these facts would be to assume that the **cation-specific P2X7R ion channel simply permeates small cations and also,** to some extent**, large cationic molecules such as cationic-dyes** (First working hypothesis in Box-1)**. However, in this case, in order to explain the permeability for large anionic molecules which is observed in certain cell types, we must admit a cell type-dependent presence of a separate P2X7R-activated permeation pathway**

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which is anion-specific (Second working hypothesis in Box-1 and the Model 1 in Figure-1, as opposed to the alternative Model 2 in Figure-1)**.** This hypothesis also explains why the Cl^- currents are observed exclusively in cells that naturally express the P2X7R (Tatham and Lindau 1990; Duan et al. 2003).

The hypothesis about a separate and cell dependent pathway for anionic-dyes is further supported by the observation that although all of the RAW 264 cells in a population uptake the cationic dye ethidium bromide, only some of them can uptake anionic dye lucifer yellow (Cankurtaran et al. 2009). This observation which was confirmed by Young and Gorecki in dystrophic myoblasts (Young and Gorecki 2018) indicates to a cell–to-cell variability across a cell population in terms of the anionic uptake phenomenon.

Mechanism(s) set aside, it is an observable fact that P2X7R induces uptake of anionic large molecules as well as cationic ones. What function, if any, this permeation serves is still a valid question. Interestingly, in macrophage-derived RAW 264 cells, the anionic-dye uptake appears to be the principal P2X7R-induced uptake response as it is much faster than the cationic-dye uptake (In Cankurtaran et al. 2009, compare the normalized YoPro-1 uptake shown in the supplements to the normalized Lucifer Yellow uptake). Here, it can be hypothesized that this anionic-pathway which can carry the anionic-dyes both ways (our unpublished results in RAW 264 cells) is a candidate for the P2X7R-activated release mechanism for the anionic neuro-transmitters such as Glutamate by astrocytes described in Duan et al. (2003) and Fellin et al. (2006) and may be considered as a separate potential drug target. Additionally, as mentioned earlier, ATP is thought to be an important mediator of inflammation and P2X7R-activated ATP^{4-} release acts as a positive feedback mechanism in inflammatory processes. This release was proposed to be through the P2X7R ionic-channel itself (Pellegatti et al. 2005, also see Di Virgilio et al. 2017), which is clearly in direct conflict with the cation-selective nature of the P2X7R pore. The P2X7R-activated separate anion-specific pathway hypothesized above may solve this problem. Whether the pannexin protein (as suggested Di Virgilio et al. 2017) or another - unidentified- protein forms this pathway still needs to be answered. Even if the role of Pannexin in cationic large molecule permeation has lost ground, its involvement in the P2X7R action as an anionic conduit is still a possibility that should be investigated. On the other hand, it is difficult to form a consistent hypothesis about P2X7R-induced ATP release for the time being, as it can

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also be seen in P2X7R-expressing HEK293 cells (Pellegatti et al. 2005) where the anionic dye uptake is not observed (Schachter et al. 2008; Cankurtaran et al. 2009). Definitely, more work is needed to clearly understand these results. Whether or not the P2X7R-induced large-cation-permeability has any function is also an interesting question that requires further investigation.

Mechanism 3. Does P2X7R Directly Interact With Cellular Signaling Pathways?

Whilst the mechanism of large molecule permeabilization had been the center of discussion, the literature contains intriguing, but mostly neglected data possibly pointing at another mechanism of action for P2X7R that seems to be distinct from the activation of permeation pathways. There are numerous studies showing that the P2X7R stimulation leads to activation of a diverse range of intracellular signaling molecules such as protein kinases or phospholipases. However, in comparison, there are very few studies designed to test whether or not the activation of these signaling molecules are due to mechanisms related to the ion channel activity of the receptor such as intracellular calcium increase, depolarization or, as discussed earlier, depletion of intracellular K^+. Yet, the existing data provide enough evidence to consider the possibility that P2X7R may not only act as an ion channel but, akin to metabotropic receptors, may directly interact with and activate intracellular signaling proteins. The well-known P2X7R-activated blebbing response, for example, which has been described both in macrophages and HEK-293 expression systems (Virginio et al. 1999a; Morelli et al. 2003; Verhoef et al 2003; Pfeiffer et al. 2004), seems to require activation of protein-kinases (Morelli et al. 2003; Verhoef et al. 2003; Pfeiffer et al. 2004; Mackenzie et al. 2005) and is independent of extracellular Ca^{2+} (Verhoef et al 2003; Pfeiffer et al. 2004), or at least has a Ca^{2+}-independent mode (Mackenzie et al. 2005). Results of Morelli et al. (2003) show that Ca^{2+} uptake is necessary but not sufficient for P2X7R-induced blebbing (Morelli et al. 2003). A receptor pharmacologically similar to P2X7R can activate PLD in BAC1.2F5 macrophages and PLA2 in submandibular glands (el-Moatassim and Dubyak 1992; Alzola et al. 1998), again without the involvement of extracellular Ca^{2+} (There is also a Ca^{2+}-dependent component of the P2X7R-induced phospholipase activation in some cells which can be discerned by using different phospholipase inhibitors (Gargett et al. 1996; Alzola et al. 1998)). Whole cell and cell-attached patch experiments in smooth muscle cells convincingly show that stimulation of a native ATP receptor with a pharmacological and electrophysiological profile similar to P2X7R (Ugur et al. 1997) activates some K^+ channels through a soluble intracellular messenger independently from K^+ efflux or Ca^{2+} influx (Zou et al. 2001). Uptake of anionic-dyes, the response

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discussed in detail above, seems to require activation of a separate pathway which is clearly not mediated by intracellular Ca^{2+} increase, or K^+ loss in RAW 264 cells (Cankurtaran et al. 2009). Similarly, an inhibitory interaction between P2X2R and Ni-AchR ($\alpha3\beta4$) which was clearly independent of factors such as Ca^{2+} influx or depolarization has also been reported in an oocyte expression system (Khakh et al 2000). Finally, P2X7R seems to interact with intracellular signal transduction proteins such as PI4K, RPTB-β or EMPs (1, 2 and 3) within large molecular complexes, The term "P2X7R signaling complex" has been coined for this proposed structure where P2X7R activity also seems to be regulated by its protein partners as a feed back mechanism (Kim et al. 2001; Wilson et al. 2002). There are also reports indicating that P2X7R may co-immuno precipitate with proteins of the NLRP inflammasome complex (Minkiewicz et al. 2013; Franceschini et al. 2015). If these findings indicate a direct interaction between P2X7R and the NLRP complex as suggested, they may add a further layer of complication to the processes such as P2X7R activated IL-1β secretion. Definitely, more studies are needed to confirm and expand these observations. Nevertheless, the available data clearly suggests that neither the ion channel activity nor the permeability to large molecules adequately explain all the actions of P2X7R and the receptor may directly interact with intracellular signaling proteins (Box-1 working hypothesis 3). Recently, similar "metabotropic"-like actions have been proposed for other well known ligand-gated ion channel receptors (for reviews see Valbuena and Lerma 2016; Kabbani and Nichols 2018).

Conclusions/Future Perspectives

In this review, we discussed examples of P2X7R-related phenomena in the framework of three working hypotheses (Box-1) with the objective of reaching a better understanding in the P2X7R field as a whole. It should be noted that, all the three hypotheses discussed in this review are working hypotheses. Although they are based on experimental results, especially the last two still require further experimental testing to be firmly established.

As the examples we discussed above illustrate, approaching the P2X7R responses from a mechanistic point of view is eventually a key factor for a clear understanding of how P2X7R works. Experiments cautiously designed to discern which of the above mechanisms is actually at work in each individual case will certainly improve and expand our vision. In our view, the most interesting and promising clues will be obtained from the experiments aimed to dissect the cation channel-related

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action mechanisms of the P2X7R (i.e. depolarization, K^+ efflux or Ca^{2+} influx) from its presumed direct interactions with intracellular signaling proteins. Clearly, careful experimental designs are required to do this dissection as both the intracellular K^+ depletion and the Ca^{2+} increase are well-established activators of cellular responses. Another equally important point here is the elimination of the contamination from other purinergic receptors (other P2Xs, more importantly P2Ys and also adenosine receptors), especially when the experiments are performed in native cells which potentially express various types of purinergic receptors. As in the case of IL-1β processing, even a seemingly simple mechanism such as K^+ depletion which is clearly P2X7R-dependent can harbor various difficulties; additional essential components such as other ion channels and other ATP receptors may come into play, creating a complicated situation. Also it should always be kept in mind that other P2X receptors may activate P2X7R-like responses, as in the case of a combination of P2X2 and P2X5 receptors which can mimic some responses of the P2X7R (Compan et al 2012).

Mechanisms of most of the P2X7R-induced responses such as promotion of cell growth, IL-1β processing and secretion, activation of anionic transport pathways, membrane blebbing are not fully understood. In addition to the examples given above, P2X7R was proposed to activate, PI3K (Amoroso et al. 2015), NF-κB (Ferrari et al. 1997b; Korcok et al. 2004), PLA2 and PLD (Panupinthu et al. 2008), AKT (Xia et al. 2015) and phosphorylates GSK3β (Amoroso et al. 2015) in various cell types. For more examples the reader is referred to Di Virgilio and Adinolfi (2015). Yet, this plethora of data, except for a few cases, gives almost no insight into the mechanisms linking the P2X7R stimulation to the activation of these proteins. These signaling proteins are known to play important roles in cancer, inflammation and many other cellular responses. The question as to whether activation of K^+, Na^+ or the Ca^{2+} fluxes is sufficient or a direct activation of intracellular signaling protein(s) is also involved in all these P2X7R-induced responses remains at the core of the P2X7R field. If the latter case is true then the next question will be the identity of these signaling proteins. In this regard, the proteins proposed to be P2X7R signaling partners in sP2X7R signaling complexes (Kim et al. 2001; Wilson et al. 2002; Minkiewicz et al. 2013; Franceschini et al. 2015) can be good candidates to start with. Resolving this point will not only drastically improve our understanding about the role of this receptor in cancer, inflammation and many other fields but may also significantly change our perception about the receptor actions in general.

Molecular Pharmacology Fast Forward. Published on February 8, 2019 as DOI: 10.1124/mol.118.115022
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Acknowledgements :

The authors wish to thank Dr. Ongun Onaran for insightful discussion and also critically reading the paper, Şule Girmen for correcting the manuscript.

Molecular Pharmacology Fast Forward. Published on February 8, 2019 as DOI: 10.1124/mol.118.115022
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions:

Wrote or contributed to the writing of the manuscript: Ugur, M. Ugur, O.

Molecular Pharmacology Fast Forward. Published on February 8, 2019 as DOI: 10.1124/mol.118.115022
This article has not been copyedited and formatted. The final version may differ from this version.

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Molecular Pharmacology Fast Forward. Published on February 8, 2019 as DOI: 10.1124/mol.118.115022
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Footnotes:

This work is sponsored by The Scientific and Technological Council of Turkey (Grant number 113S376).

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Legends for Figures:

Figure 1. Basic models to explain the available data about the P2X7R-induced cell membrane permeabilization. P2X7R protein is shown as a gray box. White boxes are proposed accessory cationic- or anionic-permeation pathways activated by P2X7R. Horizontal gray arrows indicate activation by P2X7R. YoPro and lucifer yellow are shown as representative cationic-, and anionic-large molecules respectively, as they are the commonly used experimental tracers. All the boxes represent passive transportation pathways where the actual flow directions will be dependent on the electrochemical potential of each ion. Vertical white arrows, in turn, indicate the expected flow directions of the indicated ions in commonly used experimental condition.

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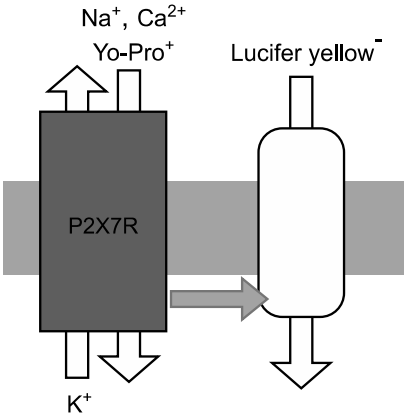
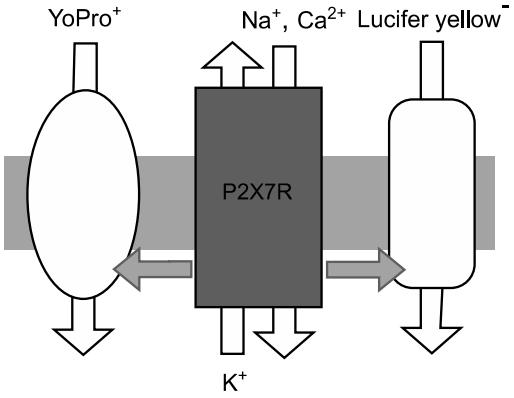


Model 1: P2X7R is a cation channel permeable to large cationic molecules. It is also coupled to an accessory pathway for anionic large molecules.



Model 2: P2X7R is a cation channel and is also coupled to (at least) two accessory pathays; one for cationic the other for anionic large molecules.