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MOL 42077

Go It Alone No More – P2X7 Joins the Society of Heteromeric ATP-Gated Receptor Channels

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Running Title: P2X4/ P2X7 Heteromeric Receptor Channels

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Abstract

P2X receptors (P2XR) function as ATP-gated non-selective ion channels permeable to Na^+, K^+, and Ca^{2+} and they are expressed in a wide range of excitable, epithelial/endothelial, and immune effector cell types. The channels are trimeric complexes composed of protein subunits encoded by seven different P2XR genes expressed in mammalian and other vertebrate genomes. Current genetic, biochemical, and/or physiological evidence indicates that the extended family of functional P2X receptors includes six homomeric channels composed of P2X1, P2X2, P2X3, P2X4, P2X5, or P2X7 subunits and six heteromeric channels that involve subunit pairings of P2X1/P2X2, P2X1/P2X4, P2X1/P2X5, P2X2/P2X3, P2X2/P2X6, or P2X4/P2X6. Thus, all P2XR subtypes – with the salient exception of P2X7R – have previously been implicated in the assembly of heteromeric ATP-gated ion channels that can comprise unique pharmacological targets in different tissues. The assumed "go-it alone" function of the P2X7R has important implications because agents that target this particular receptor have been proposed as useful therapeutics in various autoinflammatory diseases or amelioration of inflammatory pain. However, this assumption and the interpretations based on it now require reevaluation in the light of a new report in this issue of *Molecular Pharmacology* that provides convincing biochemical and electrophysiological evidence for the existence of P2X4/P2X7 heteromeric receptors.

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P2X receptors (P2XR) function as ATP-gated non-selective ion channels permeable to Na^+, K^+, and Ca^{2+}; reviewed in (North, 2002) and (Khakh and North, 2006). Most P2XR are expressed in excitable or epithelial/endothelial tissues; their ability to act as direct conduits for Ca^{2+} influx or indirect activators of voltage-gated Ca^{2+} channels underlies their multiple roles in Ca^{2+}-based signaling responses in those tissues. The channels are oligomeric complexes composed of protein subunits encoded by seven different P2XR genes (named P2X1 through P2X7 based on the order of cloning) expressed in mammalian and other vertebrate genomes. The seven P2XR subunits share a similar structure comprising two transmembrane segments, an extracellular loop containing 10 similarly spaced cysteines and glycosylation sites, and intracellular amino- and carboxy-termini. All functional P2XR subtypes display a very high selectivity for ATP over other physiological nucleotides and – with the notable exception of P2X7R – micromolar affinity for ATP (EC_{50} 1 -10 μM) (North and Surprenant, 2000). Remarkably, activation of the P2X7R requires near-millimolar concentrations of ATP ($EC_{50} \approx 300$ μM). This feature – together with its high expression and multiple roles in immune and inflammatory effector cells – has marked the P2X7R for particular attention by immunologists, electrophysiologists, and neuroscientists; reviewed in (Ferrari et al., 2006). Detailed understanding of the molecular pharmacology of these receptor channels is important because agents that target P2X7R have been proposed as useful therapeutics in various autoinflammatory diseases or amelioration of inflammatory pain (Chessell et al., 2005; Donnelly-Roberts and Jarvis, 2007; Honore et al., 2006).

Functional channels composed of P2XR subunits self-assemble during *in vivo* translation into stable, detergent-resistant trimeric complexes that traffic to the plasma

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membrane (Torres et al., 1999). When expressed separately as heterologous products in *Xenopus* oocytes or HEK293 cells, each P2XR subtype (with the exception of P2X6) can form homomeric ATP-gated ion channels. However, multiple studies have revealed that co-expression of various pairs of P2XR subtypes can yield heteromeric channels with function and pharmacology distinct from the homomeric trimers composed of either partner in the pair (North, 2002). The unique properties of natively expressed ATP-gated channels in certain tissues can be only explained by the heteromeric pairing of particular P2XR subtypes. Current genetic, biochemical, and/or physiological evidence indicates that the extended family of functional P2X receptors includes six homomeric channels composed of P2X1, P2X2, P2X3, P2X4, P2X5, or P2X7 subunits (North, 2002), and six heteromeric channels that involve subunit pairings of P2X1/P2X2 (Brown et al., 2002), P2X1/P2X4 (Nicke et al., 2005), P2X1/P2X5 (Torres et al., 1998), P2X2/P2X3 (Lewis et al., 1995), P2X2/P2X6 (King et al., 2000), or P2X4/P2X6 (Le et al., 1998). Thus, all P2XR subtypes – with the salient exception of P2X7R – have previously been implicated in the assembly of heteromeric ATP-gated ion channels that can comprise unique pharmacological targets in different tissues.

The apparent "go it alone" status of P2X7R has been a fundamental assumption in: 1) analyses of the channel functions and membrane trafficking characteristics of engineered or naturally occurring P2X7 mutant or polymorphic variants; 2) interpretation of the various physiological and pathological phenotypes in P2X7R-knockout mice; and 3) the design/ use of pharmacological agents that activate, antagonize, or allosterically modulate P2X7R. However, this assumption and the interpretations based on it now require reevaluation in the light of a report in this issue of *Molecular Pharmacology* by

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Guo et al. who provide convincing biochemical and electrophysiological evidence for the existence of P2X4/P2X7 heteromeric receptors (Guo et al., 2007). The supporting data include the usual immunoprecipitation of protein complexes containing both subunits in detergent extracts from co-transfected HEK293 cells and – very significantly – from primary murine macrophages that natively express both P2X7R and P2X4R. A particularly elegant series of studies used a point mutant (S341W) of P2X4 previously shown to assemble into homomeric complexes that traffic to the plasma membrane but lack ATP-gated channel activity (Silberberg et al., 2005). Co-expression of P2X4-S341W with P2X7 yielded ATP-gated ion currents that were markedly potentiated by ivermectin. The latter is a macrocyclic lactone used clinically to treat nematode infections but also employed by electrophysiologists as a positive allosteric modulator of various ligand-gated ion channels including homomeric P2X4R, but not other P2X-family receptors (Silberberg et al., 2007). Because ivermectin had no effect on the channel activity of P2X7R homomers, Guo et al concluded that P2X4-S341W retains the ivermectin interaction sites of wildtype P2X4 and thereby confers ivermectin sensitivity to heteromeric complexes of P2X4-S341W/P2X7 channels that are gated by the ATP-binding sites on the P2X7 subunits (Guo et al., 2007).

Interaction between P2X4R and P2X7R: A union presaged by gene duplication and overlapping patterns of expression?

From a teleological perspective, a specific interaction between P2X7R and P2X4R subunits could be presaged on the basis of several features of both gene products:

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Chromosomal location: The genes encoding human P2X7R and P2X4R are both closely located near the tip of human chromosome 12 at 12q24.31and separated by less than 24 kb (North, 2002). Similarly, the genes for murine P2X7R and P2X4R are in tandem loci only 26.46 kb apart on murine chromosome 5. These tandem chromosomal locations suggest that the P2X7 and P2X4 are related by gene duplication.

Sequence Homology: Based on the sequences of its extracellular loop and transmembrane domains, P2X4R is the most closely related P2X family member to P2X7R with 48.6% pairwise amino acid identity (for human P2X7R and P2X4R). This contrasts with the 41-45% identities between P2X7R and the other P2XR subtypes (North, 2002).

Co-expression in multiple non-excitatory tissues and cells: The overlapping expression of P2X4R and P2X7R mRNA, protein, and presumed homomeric channel activity has been documented in multiple tissues and non-excitatory cell types including epithelial cells from salivary glands (Tenneti et al., 1998), exocrine pancreas (Hede et al., 1999), and airways (Korngreen et al., 1998; Zsembery et al., 2003), myeloid-lineage leukocytes that include macrophages (Guo et al., 2007) and microglial cells (Raouf et al., 2007), and osteoclasts (Korcok et al., 2004) . Patch-clamp analysis of ATP-gated gated inward currents (I_{ATP}) in these cell types has generally indicated biphasic relationships between I_{ATP} and ATP concentration with one set of currents stimulated by micromolar ATP and a second group currents triggered in response to millimolar ATP.

Overlapping roles of P2X7R and P2X4R in inflammatory pain signaling: Using a Freund's complete adjuvant (FCA)-injected paw model of inflammation, Chessel *et al*. found that local IL-1β levels in the inflamed paws of P2X7R-null mice were reduced 2.5-

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fold at 1 day, and 5.4-fold at 7 days post-FCA injection with no global changes in serum IL-1β. (Chessell et al., 2005) Moreover, the hypersensitivity to thermal or mechanical stimuli (*i.e.* allodynia or inflammatory pain) that characterizes the inflamed paws of control mice was completely absent in the P2X7R-knockout animals. Similarly, Inoue and colleagues used a spinal cord injury model to show that pharmacological or anti-sense suppression of P2X4R signaling in microglial cells markedly decreased the mechanical allodynia that accompanies this type of nerve injury (Tsuda et al., 2003). Thus, both P2X4R and P2X7R play overlapping roles in inflammatory nociception and have been considered as potential therapeutic targets in this condition.

Interaction between P2X4R and P2X7R: Implications and questions for future study

This newly identified ability of P2X7R to interact with P2X4R has multiple implications regarding current understanding of P2X7R-based functional responses and also generates significant questions for future study.

Regulation of secondary P2X7R signaling responses by P2X4/P2X7 heteromers?: Homomeric P2X7R channels and homomeric P2X4R channels both trigger the common depolarization and Ca^{2+} influx responses that typify all subtypes of ATP-gated ion channels. However, many studies have demonstrated that P2X7R additionally elicits a wide range of secondary signaling responses more typically associated with G protein-coupled receptors than ligand-gated channel receptors (**Figure 1**). Activation of these downstream signaling pathways by P2X7R in myeloid or lymphoid leukocytes likely underlies the ability of this receptor to shape the intensity or duration of innate immune and inflammatory responses (Ferrari et al., 2006). Although gating of cation channel

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function is the most immediate consequence of ATP-induced changes in P2X7R conformation, increasing evidence suggests that the P2X7R additionally acts as a docking site for multiple intracellular proteins. Surprenant and colleagues used proteomic analysis of anti-P2X7R immunoprecipitates to demonstrate P2X7R association with various signaling and cytoskeletal proteins (Kim et al., 2001). Moreover, sustained stimulation of P2X7R additionally induces the flux of molecules ≤ 800 Da through an indirectly regulated non-specific porin recently been identified pannexin-1 (Pelegrin and Surprenant, 2006). P2X7R also activates the small GTPase Rho that can activate phospholipase D, Rho-effector kinases (ROCKs) and ROCK-dependent membrane blebbing (el-Moatassim and Dubyak, 1992; Verhoef et al., 2003). The massive increase in Ca^{2+} induced by P2X7R elicits a rapid flip of phosphatidylserine (PS) to outer leaflet of the plasma membrane similar to that observed in apoptotic cells (Mackenzie et al., 2005). The P2X7 receptor is significantly larger (595 amino acids) than all other P2X subtypes due to its much longer intracellular C-terminus. This unique C-terminal tail appears to contain the molecular determinants for induction of the various secondary signaling responses including the pannexin-mediated change in membrane permeability (North, 2002). In contrast, the cytosolic C-terminus of P2X4R is the shortest among P2XR subtypes (**Figure 1**) such that the juxtaposed intracellular domains of a P2X4R homotrimer are less likely to constitute an effective platform for recruitment of various signaling proteins or interaction with pannexins. However, depending on their stoichiometry, P2X4R/P2X7R heteromers will contain one or two P2X7R-derived C termini which may be sufficient for recruitment of at least a subset of the downstream signaling proteins that shape the integrated cellular responses to P2X7R stimulation.

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Dynamic regulation of P2X4R/P2X7R heteromer copy number and P2X4R/P2X7R heteromer stoichimetry: The expression of both P2X7R and P2X4R is known to be regulated during proinflammatory activation of monocyte/ macrophages (Humphreys and Dubyak, 1998), microglia (Raouf et al., 2007), and endothelial cells (Ramirez and Kunze, 2002; Wilson et al., 2004) . P2X7R is strongly expressed in resting macrophages and microglia while P2X4R expression is modest. However, activation of toll-like receptor receptors (TLR) by LPS or other inflammatory stimuli markedly up-regulates P2X4R while not altering P2X7R levels (Raouf et al., 2007). Conversely, there is high P2X4R expression (Yamamoto et al., 2006) and only low to no P2X7R expression (Beigi et al., 2003) in non-inflamed vascular endothelial cells (EC) ; P2X7R is increased when EC are exposed to inflammatory cytokines (Wilson et al., 2004). These observations suggest that the relative copy numbers of both P2X7R and P2X4R subunits will change in these various cell types at different stages of inflammatory activation. Thus, future studies will need to test how these alterations in P2X7R and P2X4R subunit levels dynamically regulates their relative distribution in P2X7 homomers, P2X4 homomers, and the two possible types of P2X4R/P2X7R heteromeric channels.

Modulation of P2X7-based phenotypic responses in P2X4-knockout mice: Finally, a related set of issues pertains to whether certain altered phenotypes observed in P2X7R-knockout mice might actually involve the specific absence of P2X4R/P2X7R heteromers versus P2X7R homomeric channels. These phenotypes include reduced progression of anti-collagen induce arthritis (Labasi et al., 2002), change in bone density during aging (Ke et al., 2003), and processing of inflammatory pain stimuli (Chessell et al., 2005). The recent availability of several P2X4R-knockout mouse lines should facilitate future

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experiments that test whether the absence of P2X4R recapitulates or additionally modulates these phenotypic changes associated with P2X7R deletion (Sim et al., 2006; Yamamoto et al., 2006).

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Figure Legend

Figure 1. Membrane topology and signaling functions of P2X7R homomers, P2X4R homomers, and P2X4R/P2X7R heteromers

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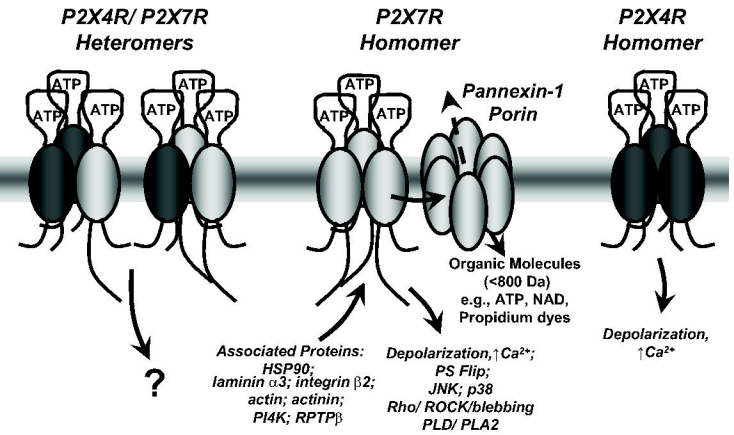
P2X4R/ P2X7R
Heteromers
P2X7R
Homomer
P2X4R
Homomer
ATP
Pannexin-1
Porin
Organic Molecules
(<800 Da)
e.g., ATP, NAD,
Propidium dyes
Depolarization,
↑Ca²⁺
?
Associated Proteins:
HSP90;
laminin α3; integrin β2;
actin; actinin;
PI4K; RPTPβ
Depolarization,↑Ca²⁺;
PS Flip;
JNK; p38
Rho/ ROCK/blebbing
PLD/ PLA2