Highlights in Purinergic Signalling

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Chaumont S, Compan V, Toulme E, Richler E, Housley GD, Rassendren F, Khakh BS (2008) Regulation of P2X2 receptors by the neuronal calcium sensor VILIP1. *Sci Signal* 1(41):ra8. doi: 10.1126/scisignal.1162329

Article summary

It has become increasingly clear that receptors and ion channels in the plasma membrane do not exist and function in isolation, but instead form complexes with other proteins and this can modify their expression and activity. In this study, the authors determined which proteins complex with the P2X2 receptor and characterised the influence of one, visinin-like protein 1 (VILIP1) on P2X2 receptor expression and activity. They found that P2X2 receptors are part of a complex with nine other proteins and that through a constitutive interaction VILIP1 selectively increased P2X2 receptor expression at the plasma membrane threefold, increased the amplitude of ATP-induced currents by a similar amount and prolonged the life-time of the P2X2 receptor in the plasma membrane. Activation of the P2X2 receptors by ATP increased the association between the two proteins via a conformational change in the P2X2 COOH tail and this effect was dependent on Ca^{2+} influx through the P2X2 pore. This Ca^{2+} binds to and most likely causes a conformational change in VILIP1 as well. Thus, the neuronal Ca^{2+} sensor VILIP1 acts as a regulator of P2X2 receptors.

Commentary

Historically, receptors in the plasma membrane were viewed in a simple manner: ligand-gated ion channels were “stand alone” functional units, which opened when bound by an agonist, to allow ion flux across the plasma membrane, whilst binding of an agonist to G protein-coupled receptors (GPCR) caused them to interact with an intracellular G protein, which in turn interacted with intracellular signalling pathways. However, it has become apparent that the expression and activity of receptors is in fact much more complex and that receptors are clearly only one component in a multi-protein complex that includes scaffolding proteins, regulatory proteins, kinases and phosphatases. Furthermore, receptor expression and activity is greatly influenced or modulated by the other proteins in the complex. For example, an early study found that the NMDA receptor is part of a complex comprising more than 70 proteins (Husi H. et al., *Nat Neurosci* (2000) 3: 661-669) and many other receptors and ion channels have since been shown to form similar complexes (Levitan IB, *Nat Neurosci* (2006) 9: 305-310).

The present study shows that P2X receptors, in common with other major receptor and ion-channel families, are part of functionally active protein complexes. Of particular interest was VILIP1, as it is a Ca^{2+} sensor that is thought to modulate neuronal activity and which has a similar distribution to P2X2 receptors in many areas of the brain. Indeed, using specific antibodies they found colocalisation of P2X2 receptors and VILIP1 in mouse deep cerebellar nuclei and the dentate gyrus, though not in cerebellar Purkinje and granule cells. Thus, these two proteins are clearly closely associated in certain areas of the brain. Furthermore, when the two proteins were coexpressed in hippocampal neurones, action potentials induced release of vesicular ATP, which in turn activated the P2X2 receptors and caused them to interact more closely with VILIP1.

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Fluorescence resonance energy transfer analysis indicated that individual P2X2 receptors and VILIP1 molecules in the plasma membrane are situated 60 Å apart, which is close enough for them to interact physically, though at present, the number of VILIP1 molecules that interact with a single, trimeric P2X2 receptor is unknown. Also, it is uncertain if P2X2 receptor activation recruits further VILIP1 molecules to the protein complex or if all complexes are preformed. Finally, the roles of the other proteins identified in this complex (vacuolar-type H⁺ ATP-ase, heat-shock protein 90, *N*-ethylmaleimide-sensitive factor, vesicle amine transport protein 1 homologue, tubulin 1 α , glutamine synthase, glutamic acid decarboxylase and synapsin IIb) and their influence, if any, on P2X2 receptor expression and activity remain to be determined. Nonetheless, this study greatly improves our understanding of how P2X receptors form complexes and interact with other cellular proteins, and how their expression and activity is modulated by these proteins.

Norambuena A, Poblete MI, Donoso MV, Espinoza CS, González A, Huidobro-Toro JP (2008) P2Y₁ receptor activation elicits its partition out of membrane rafts and its rapid internalisation from human blood vessels: implications for receptor signalling. *Mol Pharmacol* 74:1666-1677.

Article summary

Rafts are microdomains of the plasma membrane that are a complex of lipids and proteins and in which receptors and ion channels are often concentrated. The authors showed that a significant proportion of the total cellular P2Y₁ receptors in human chorionic artery smooth muscle cells was present in plasma membrane raft domains and that this association is essential for agonist-induced vasoconstriction. Disruption of the rafts by a cholesterol-depleting agent had no effect on the total cellular expression of P2Y₁ receptors, but decreased their expression in rafts by 70% and also inhibited the vasoconstriction induced by the P2Y₁ agonist 2-methylthioADP, although contractions to potassium chloride and 5-hydroxytryptamine were unaffected. In addition, receptor activation regulates this localisation, as the P2Y₁ agonists ADP, 2-methylthioADP caused the receptor to move out of the rafts and this correlated with the fade in the agonist-induced vasoconstriction.

Commentary

The plasma membrane was for many years thought to comprise a homogeneous bilayer of lipids in which proteins such as receptors and ion channels floated like icebergs. However, it is now known that it has in fact a heterogeneous structure and that lipid or membrane rafts consisting of a complex of glycosphingolipids, cholesterol and

structural proteins, such as flotillins and caveolins, form separate compartments that concentrate (or exclude) particular receptors and ion channels (Pike LJ, *Lipid Res* (2006) 47: 1597-1598). These rafts regulate various important physiological processes, including the trafficking of receptors and their interaction with intracellular signalling pathways, including P2 receptors (Garcia-Marcos M. et al., *FEBS J* (2008) 276: 330-340).

The present study shows clearly that P2Y₁ receptors in human chorionic arteries partition into membrane rafts and that this localisation is essential for the functional activity of the receptor. Nearly 20% of the total cellular P2Y₁ receptor comigrated with the raft components flotillin 1, caveolin 1, caveolin 3 and with G α_q , the G protein through which the P2Y₁ receptor signals. Similar levels of association were also found in smooth muscle cells of human mammary arteries and saphenous veins. Rafts are rich in cholesterol and so can be disrupted by treatment with cholesterol-depleting agents. This procedure greatly decreased P2Y₁ receptor presence in rafts and crucially, it virtually abolished P2Y₁ receptor-mediated vasoconstriction. These effects were only slowly reversible once the receptor fully reassociated with the rafts and the subsequent contractions and associated receptor internalisation were much smaller than before. Why this was so and how it relates to exposure of the tissue to ADP under physiological conditions is unclear. Also, the mechanism underlying P2Y₁ receptor internalisation and the fate of the internalised receptors remain to be determined. Surprisingly, whilst the P2Y₂ receptor was also present in chorionic artery smooth muscle cells, it was not present in the rafts. How this exclusion of P2Y₂ receptors influences their expression and functional activity was not studied further.

Interestingly, the importance of rafts in P2Y₁ receptor signalling appears to be tissue-specific, as disruption of rafts in human platelets had no effect on the rise in intracellular [Ca²⁺] mediated via P2Y₁ receptors, although it did substantially inhibit that evoked via P2X₁ receptors (Vial C. et al., *Biochem Biophys Res Comm* (2006) 343: 415-419). The functional activity of P2Y₁₂ receptors in platelets also appears to depend upon their compartmentalisation into rafts, since the active component of the antithrombotic drug clopidogrel, which is a P2Y₁₂ antagonist, inhibited platelet aggregation and caused the receptor to move out of the rafts (Savi P. et al., *Proc Natl Acad Sci USA* (2006) 103: 11069-11074). Thus, membrane rafts play an important role in regulating P2 receptor expression and function in a tissue-specific manner.

Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ (2008) Agonist-selective, receptor-specific interaction of human P2Y receptors with β -arrestin-1 and -2. *J Biol Chem* 283:30933-30941.

Article summary

The activity and expression of receptors can be modified not only by other members of the plasma membrane complex, but also by cytosolic proteins that translocate to the plasma membrane upon receptor activation. The authors tagged six of the eight currently known human P2Y receptors (the $G\alpha_q$ -coupled P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ receptors and the $G\alpha_{i/o}$ -coupled P2Y₁₂ receptor) and the regulatory proteins β -arrestin-1 and 2, with fluorescent markers and found that activation of each of the six P2Y subtypes cause translocation of β -arrestins from the cytosol to the plasma membrane. Furthermore, apart from the P2Y₁₂ receptor, they all underwent rapid internalisation when stimulated, although coexpression of a G protein receptor-coupled kinase was necessary in some cases. Internalisation for all, except the P2Y₂ receptor, was via a dynamin-dependent process. Finally, perhaps the most interesting result in the present study was that the two endogenous agonists, ATP and UTP, caused the P2Y₂ receptor to interact differentially with β -arrestin-1 and 2 and this was reflected in different time courses for increased phosphorylation of the downstream target, ERK.

Commentary

The cytosolic proteins β -arrestin-1 and 2 are important components of the cellular machinery that regulates GPCR desensitisation and internalisation (Lefkowitz RJ, Shenoy SK. *Science* (2005) 308: 512–517). On binding of an agonist, GPCR can be phosphorylated by the second messenger-activated kinases or by G protein-coupled receptor kinases. The latter effect causes β -arrestins to bind to the phosphorylated GPCR, which in turn causes the receptor to uncouple from its associated G protein. Counter-intuitively, this can lead to initiation of intracellular signalling, such as phosphorylation of the enzyme extracellular-signal regulated kinase (ERK), in a β -arrestin-specific manner. Thus, receptors need not even be present in the plasma membrane in order to display functional activity and GPCR, such as β -adrenoceptors, can continue to activate cellular signalling pathways even after they have been internalised.

In the present study, activation of both $G\alpha_q$ - and $G\alpha_{i/o}$ -coupled P2Y receptors caused β -arrestin to move from the cytosol to the plasma membrane, which in turn caused the receptors to move from the plasma membrane to the cytosol. Receptor internalisation is often dependent upon dynamin and coexpression of rat dynamin 1a appeared to increase agonist-induced internalisation of each of the six

P2Y subtypes, whereas coexpression of a dominant-negative mutant of dynamin, Dyn-K44A, inhibited it for all, except the P2Y₂ receptor. The mechanism by which the P2Y₂ receptor internalises was not studied further. How these results with recombinant receptors expressed in a cell line relate to the interaction of native receptors *in situ* with β -arrestins and their internalisation is of great interest, though this is hampered by the relatively small number of highly subtype-selective, P2Y receptor antibodies that are currently available.

A further level of signalling complexity was revealed when it became apparent that a single receptor can couple to different G proteins and signalling pathways in an agonist-dependent manner. For example, the coupling of the human D₂-dopamine receptor to the $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{o1}$ G proteins varies with the agonist used (Gazi L. et al., *Br J Pharmacol* (2003) 138: 775–786).

The present study showed that the endogenous agonists ATP and UTP appear to induce different conformational changes in the P2Y₂ receptor, which causes it to interact with the β -arrestins in a different manner. UTP induced similar translocation of β -arrestin-1 and 2, whereas ATP caused greater movement of β -arrestin-1 than β -arrestin-2. These differential effects of ATP and UTP had a downstream effect on cell signalling as both agonists increased the levels of phosphorylated extracellular-signal-regulated kinase, but the effect of UTP was transient, whereas that of ATP was prolonged. This is contrary to the classical model of receptor activation, but agrees with an increasing appreciation that receptors are not simple on–off switches, but instead undergo multiple, agonist-induced changes in conformation. Thus, P2Y receptors are at the cutting edge of this aspect of GPCR pharmacology.

About the Author

Dr. Charles Kennedy is presently a Reader at the Strathclyde Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde, Glasgow, Scotland. His research interest relates to (1) the characterization and classification of P2X and P2Y receptors, (2) the role of ATP and neuronal P2X₃ receptors in mediating certain types of pain, (3) the role of purines in neurotransmission, and (3) the modulation of purinergic neurotransmission by ecto- and soluble nucleotidases. He has been a member of the IUPHAR P2X and P2Y receptor nomenclature sub-committees for many years and became chair of the P2X sub-committee at the beginning of 2009.