A Dual Polybasic Motif Determines Phosphoinositide Binding and Regulation in the P2X Channel Family

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Abstract

Phosphoinositides modulate the function of several ion channels, including most ATP-gated P2X receptor channels in neurons and glia, but little is known about the underlying molecular mechanism. We identified a phosphoinositide-binding motif formed of two clusters of positively charged amino acids located on the P2X cytosolic C-terminal domain, proximal to the second transmembrane domain. For all known P2X subtypes, the specific arrangement of basic residues in these semi-conserved clusters determines their sensitivity to membrane phospholipids. Neutralization of these positive charges disrupts the functional properties of the prototypical phosphoinositide-binding P2X4 subtype, mimicking wortmannin-induced phosphoinositide depletion, whereas adding basic residues at homologous positions to the natively insensitive P2X5 subtype establishes de novo phosphoinositide-mediated regulation. Moreover, biochemical evidence of in vitro P2X subunit-phospholipid interaction and functional intracellular phosphoinositide-binding assays demonstrate that the dual polybasic cluster is necessary and sufficient for regulation of P2X signaling by phospholipids.

Introduction

ATP-gated P2X receptor channels play significant roles in pain transduction, neuro-immune interactions and inflammatory response therefore understanding their regulation mechanisms is critical. Plasma membrane phosphoinositides (PIP₃) are anionic phospholipids that act as functional regulators of many types of ion channels. They are necessary cofactors for activation or desensitization of various channels, including transient receptor potential (TRP) channels [1,2], inward rectifier K⁺ (Kir) channels [3,4] and voltage-gated KCNQ channels [5]. Most ATP-gated P2X receptor subtypes are potentiated by intracellular PIP₃: P2X1, P2X2, P2X3, P2X4 and P2X7 are functionally sensitive to PIP₃ [6,7,8,9], however, P2X5 was found to be PIP₃-insensitive [10].

In P2X subunits, the few residues shown to be implicated in PIP₃-mediated regulation are located in the C-terminal domain, which is also involved in subunit trafficking, phosphorylation, heteromerization and multi-receptor crosstalks [11,12,13,14].

Although no consensus PIP₃ binding site exists among membrane proteins, analysis of the PIP₃-binding region of PH domain-containing proteins points to the necessary presence of basic amino acids interacting with the anionic headgroup of PIP₃ [15]. The identity of PIP₃-binding domains in ion channels in particular has been even more elusive, with only a few putative residues identified as involved in the interaction, all on the cytoplasmic side of the membrane [for review [16]]. Although direct protein-phospholipid binding was demonstrated for several families of PIP₃-sensitive channels, no common motif can predict an effective interaction.

Here, we demonstrate that P2X channel subunits bind to PIP₃ via two clusters of positively charged residues located in the proximal C-terminal domain. The specific arrangement of basic and acidic amino acids found in these semi-conserved clusters predicts the PIP₃-sensitivity of all known P2X subtypes. By mutating the prototypical PIP₃-sensitive P2X4 and PIP₃-insensitive P2X5 subtypes, we provide functional and biochemical evidence that a dual cluster motif in the proximal C-terminal domain is necessary and sufficient for the regulation of P2X receptor channels by PIP₃.

Results

A PIP₃-binding Site in P2X C-terminal Domains

Several studies have demonstrated functional modulation of P2X receptors by PIP₃ as well as direct PIP₃ binding to the C-terminal domain of various P2X subunits (Figure 1A, left columns) [6,7,8,9,10,17]. P2X1, P2X2 and P2X4 subunits directly bind PIP₃, whereas P2X3 and P2X5 do not. Hence, we analyzed their respective C-terminal sequences and found that PIP₃ binding correlates with the net positive charge of two polybasic amino acid clusters (Figure 1, shaded areas 1 and 2). P2X1/2/4 contain 6–7 basic residues (lysine, arginine or histidine) in these two clusters and a maximum of one negatively-charged residue (aspartic or glutamic acid). On the other hand, the non PIP₃-binding P2X3 and P2X5 subunits contain 5 and 6 basic residues, respectively, but also 3 acidic residues disrupting the global positive charge of the clusters. We therefore hypothesized that the dual cluster’s charge is responsible for the affinity of the P2X C-terminus to
A Dual Polybasic Cluster Motif is Necessary for PIP$_n$-binding and P2X4 Channel Regulation

We reported that P2X4 is a prototypical PIP$_n$-dependent P2X subtype, being tightly regulated via direct binding to PIP$_2$ and PIP$_3$ [8]. We therefore aimed to neutralize the PIP$_n$-binding site by mutating key lysine residues. We found that neutralizing the charge of either of the two clusters, by mutating lysines 362 and 363 or lysines 370 and 371 into neutral glutamines, leads to a loss of PIP$_n$ binding in an in vitro binding assay where a GST-fusion protein coding for a 16-amino acid sequence (Figure 2A,B) is applied to various PIP$_n$. The lysine-to-glutamine mutations performed on residues 362 and 363 also induced significant changes in the P2X4 channel activity. Expressed in the *Xenopus* oocyte expression system, the P2X4 mutant with lower PIP$_n$-binding is also significantly less functional than the wild-type receptor.

**Figure 1.** The proximal C-terminal domain of P2X subunits contains a semi-conserved PIP$_n$-binding motif. A) Sequence alignment of rat P2X C-termini proximal to the TM2 domain showing the two polybasic clusters (shaded area, 1 and 2). The left column summarizes, for each subunit, the presence (+) or absence (−) of binding of the GST-fusion C-terminal domain to PIP$_n$ in PIP strip assays. The second column shows the presence (+) or absence (−) of modulation by PIP$_n$ in functional assays. Basic residues are shown in red and acidic residues in blue. B) Sequences showing residues that were reported (here or previously) to be involved in PIP$_n$ regulation. Basic residues in red, acidic residues in blue and an uncharged serine in green. C) Schematic representation of the topology of a P2X subunit showing binding of two positively charged amino acid clusters to membrane-bound PIP$_n$.
binding affinity displayed a stronger current rundown upon repeated ATP applications as well as slower activation and desensitization current phases (Figure 2C,D), all these effects mimicking those brought by pharmacological PIP₃ depletion [8]. The K362Q-K363Q mutant receptor was more strongly inhibited by wortmannin-induced PIP₃ depletion than the wild-type (WT) receptor (Figure 2E), due to its lower affinity to PIP₃. The mutation targeting the second basic cluster (K370Q-K371Q) could not be tested functionally as P2X4 channels with mutations on residue 371 are non-functional due to the role of conserved lysine 371 in receptor trafficking [11].

P2X1 and P2X7 Binding to PIP₃ is Consistent with the Dual Polybasic Cluster Model

For the P2X1 subtype, the results that we have previously reported are consistent with our model, in that mutating the K359 residue in the first cluster suppressed in vitro binding to PIP₃, and induced a PIP₃-depleted like current phenotype [7]. To confirm that both clusters are involved in the interaction with PIP₃, we neutralized the charge in the second cluster via a lysine-to-arginine mutation (Figure 3A), confirming that both clusters are necessary for the P2X1 C-terminus to bind PIP₃.

The P2X7 subtype was also analyzed, but no direct binding was found in our biochemical binding assay using C-terminal peptides of various length (Figure 3B). The absence of binding is likely due to the presence of only one polybasic cluster in the P2X7 C-terminus. Nevertheless, a previous report demonstrates through a mutational study that specific amino acids are involved in PIP₃ modulation of P2X7 [17], suggesting a more complex binding mechanism, likely due to the presence of an additional 18-residue long sequence between the cluster and TM2.

Generation of De Novo PIP₃-regulation in the P2X5 Subtype

To verify if the presence of C-terminal polybasic clusters is sufficient for PIP₃ regulation of P2X receptor channels, we chose the native PIP₃-insensitive P2X5 subunit and examined the effect of adding basic residues to its C-terminus (Figure 4A,B). In the phospholipid binding assay, adding positive charges to the cluster proximal to TM2 by mutating residues 365 and 366 induced binding to several PIP₃. Also, mutating a negatively-charged glutamic acid into a lysine in the second cluster enhanced binding, thereby showing that PIP₃ binding can be obtained via negative-to-positive mutations in the twin clusters (Figure 4C). We then analyzed the functional effect of that mutation by recording from the S365K-E366Y-E374K mutant in the Xenopus oocyte expression system: the mutant P2X5 receptor generated currents ~15 times larger than the WT upon 10 µM ATP activation. Adding basic residues to the first cluster only (S365K-E366Y) also led to significantly larger currents than the WT. Whereas WT P2X5 is unaffected by wortmannin treatment, both PIP₃-binding mutants were strongly inhibited by wortmannin-induced PIP₃ depletion, suggesting that PIP₃ binding is responsible for the current amplitude increase induced by the C-terminal mutations (Figure 4B,D). WT P2X5 channels display a marked current rundown upon repeated activation; such a feature was absent in the triple mutant, but could be restored after pharmacological PIP₃ depletion (Figure 4F). Also, the activation rate of the P2X5 current was faster in the PIP₃-binding mutant than in the WT, as was the desensitization rate. Both properties were restored towards WT levels after wortmannin treatment, confirming the PIP₃-sensitivity of the mutant receptor channel.

The human P2X5 ortholog has an arginine residue on position 365 and has been shown to evoke currents of much larger amplitude than its rat homolog [19], we therefore verified if PIP₃ play a role in this difference. Wortmannin-induced PIP₃ depletion significantly reduced the hP2X5 current amplitude (Figure 4E), indicating that interspecies differences exist in terms of functional PIP₃ regulation of P2X3 channels and confirming the importance of the positive charges found in the proximal polybasic cluster.

P2X4 C-terminal Peptides Compete for Intracellular PIP₃ and Induce a PIP₃-depletion Current Phenotype

To confirm that P2X C-terminal polybasic clusters bind to PIP₃ in a cytoplasmic environment, we performed an intracellular PIP₃-binding competition assay. H.E.K293 cells transiently expressing P2X4 were recorded in whole-cell patch-clamp configuration, and various GST fusion proteins containing the P2X C-termini (6 amino acid-long, P2X4: C360-V375, P2X5: L361-V376) were added to the intracellular milieu. When PIP₃-binding P2X4 C-terminus peptides were introduced through the patch pipette, a strong rundown of the ATP-mediated P2X4 current as well as a strong decrease in desensitization rate were observed, suggesting that the P2X4 C-terminal peptide competes for intracellular PIP₃ binding, inducing a PIP₃-depletion current phenotype (Figure 5A,C,D). Peptides coding for the P2X4 K362Q-K363Q mutant C-terminal domain did not induce any change in the current phenotype as compared to a control GST peptide injection, indicating an inability to bind intracellular PIP₃.

Reciprocally, the WT P2X5-C-terminus peptide had no effect on both functional parameters measured (Figure 5B,C,D) due to its low PIP₃-binding affinity. Strong interactions between the P2X5 S365K-E366Y-E374K mutant peptides and PIP₃, led to rundown and slower desensitization of the P2X4 currents.

Discussion

Membrane PIP₃ regulate the activity of a wide variety of ion channels, and the mechanism of interaction between these important membrane proteins and the anionic phospholipids draws lots of attention. We show here that the modulation of P2X channel function by PIP₃ is predicted by the subunit’s ability to bind to the negative inositol trisphosphate head group of the lipid via two adjacent clusters of basic amino acids located on the C-terminal domain. Binding of PIP₃ to the clusters present in P2X1, P2X2 and P2X4 likely leads to a conformational change in the C-terminus, a domain highly involved in functional regulation of the channel.

The P2X3, P2X5 and P2X7 subunits lack this microdomain and therefore do not directly interact with PIP₃. While the absence of this microdomain renders P2X5 channel activity insensitive to PIP₃, P2X3 and P2X7 are functionally modulated by PIP₃ [6,17], strongly indicating an indirect regulation. A mechanism in which a PIP₃-binding partner protein acts as a regulatory subunit has been proposed for TRPV1, where phosphoinositide interacting regulator of TRP (Pirt) is necessary for PIP₃ mediated enhancement of the channel activity [20]. Pirt, a membrane protein which binds PIP₃ via a cluster of basic residues on its C-terminus, also complexes with TRPV1 to link both molecules. A similar interaction was observed in the case of NMDA receptors, where the cytosolic tails of the NR1 or NR2B subunits bind α-actinin, an actin-crosslinking protein. α-actinin also binds membrane PIP₂ and modifies the NMDA receptor’s intracellular tail conformation to promote channel opening [21]. A similar mechanism could underlie the indirect PIP₃-dependent regulation of P2X3 and P2X7, but the nature of the partner involved remains to be elucidated.
Interestingly, P2X7 forms a signalling complex with various proteins that includes α-actinin [22], which could link the P2X7 C-terminal tail to PIP₃.

Proteins bind PIP₃ via multiple contacts that require the contribution of multiple amino acids [16], hence we analyzed the general charge of the P2X C-terminal domain, and found that the 13-amino acid sequence containing both clusters has a predicted isoelectric point of 10.4 to 10.8 for P2X1/2/4/5, but that P2X3 and P2X7 have lower predicted values of 9.2 and 8.5, respectively. This could explain the lack of direct PIP₃ binding in these two subunits. However, PIP₃-sensitive P2X1/2/4 and PIP₃-insensitive P2X5 subunits have similar isoelectric points, suggesting that PIP₃...
Figure 3. Requirement of two polybasic clusters for PIP_n-binding in P2X1 and P2X7. A) The GST construct containing the WT P2X1 C-terminal domain (L352-E378) (basic residues in red, acidic in blue, neutral mutations in grey) binds various PIP_n on a phospholipid strip assay, whereas disrupting the positive charge of the first or second polybasic cluster with K359Q and K364Q mutations suppresses binding (n = 3). B) The absence of two polybasic clusters in the C-terminus of P2X7 prevents its binding to PIP_n on a phospholipid strip assay (n = 3). Shown in grey boxes are various GST-fusion peptides generated.

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The gating mechanism underlying ion conduction in P2X channels has been extensively studied in recent years. It is believed that the gate is located between residues 340 to 347 (nomenclature for zP2X4.1) in the TM2 domain, and that opening of the channel triggers rearrangement of the TM2 helices that reveals access to even deeper parts of the pore a few residues away from the cytoplasmic tail [29,30,31]. The PIP_n-binding region therefore lies in close proximity to the gating machinery of P2X receptor channels, likely impacting on the open/closed transition through conformational changes.

Results obtained on the P2X4 and P2X5 subunits not only enhance our understanding of the PIP_n-binding site of P2X receptors, but also demonstrate the importance of PIP_n in the functional regulation of the channels. Disrupting the PIP_n-affinity of P2X4 led to major changes in the current phenotype, similar to what was seen in other PIP_n-binding P2X subtypes [7,9]. Moreover, we were able for the first time to induce a PIP_n-binding phenotype through single mutations in the otherwise PIP_n-insensitive P2X5 subtype, demonstrating that the polybasic clusters motif is sufficient for PIP_n binding and functional regulation. The high amplitude currents obtained with the gain-of-binding mutant suggest that the small size of currents mediated by the WT rat P2X5 in several expression systems [10,32] is due to its low PIP_n-affinity. Altogether, our results indicate that membrane PIP_n contribute to the full expression of P2X receptor channel function.

Interestingly, it was shown that the human and chicken P2X5 receptors give rise to currents that are significantly larger, and desensitize faster than their rat counterparts [19,33]. Analysis of their C-terminal sequence shows that human P2X5 has a basic arginine residue on position 365, instead of a neutral serine found in the rat sequence, and that chicken P2X5 has a neutral asparagine on position 366, instead of a negatively-charged glutamic acid in the rat sequence. Our results demonstrate that a neutral-to-basic mutation on residue 365 and an acidic-to-neutral mutation on residue 366 induces a PIP_n-binding phenotype in the rat P2X5. We also show that the human P2X5 channel is regulated by PIP_n as pharmacological depletion of intracellular PIP_n induced dramatic changes to its current phenotype. It has to be noted that on position 375, at the extremity of the second
Figure 4. Mutations creating two polybasic clusters on the P2X5 C-terminus lead to PIP₃ binding and a PIP₃-regulated current phenotype. A) Sequence of the P2X5 C-terminus showing mutations adding positive charges to one (S365K-E366Y, SE→KY) or two clusters (S365K-E366Y-E374K, SEE→KYK) (basic residues in red, acidic in blue, neutral mutations in grey). B) Representative ATP-activated current traces recorded in *Xenopus* oocytes expressing P2X5 WT or SEE→KYK mutant in control and wortmannin conditions. C) The GST construct containing the WT P2X5 Phosphoinositide-Binding Motif in P2X Family.
polybasic cluster, a lysine is found in the rat sequence instead of a glutamic acid in the human and chicken orthologs. This suggests that the first cluster plays a preponderent role in PIP₆ binding, in agreement with the gain-of-binding S365K-E366Y mutation performed on rat P2X5. Since mutations increasing the PIP₆-binding affinity of P2X5 have a drastic effect on its ion channel function, it is likely that these differences in C-terminal sequence account for the high degree of variability of P2X5 phenotypes observed among vertebrate species.

Identification of the molecular determinants of PIP₆-protein interactions in the P2X family confirmed the intrinsic and essential nature of PIP₆ regulation of P2X channel activity. Knowing that intracellular PIP₆ levels are controlled by a wide array of ubiquitous pathways such as Gq-coupled receptor-induced phospholipase C hydrolysis of PIP₂ or receptor tyrosine kinase activation of PI3K, the P2X-PIP₆ regulatory mechanism is likely involved in multi-receptor crosstalks. Our predictive model unifies various data obtained on PIP₆-regulation of P2X ion channels.

Materials and Methods

Two-electrode Voltage-clamp Recordings in Xenopus Oocytes

Oocytes were removed from *Xenopus laevis* frogs as described [8] before intranuclear microinjection of 1 ng plasmid DNA coding for rat P2X4 (WT or K362Q-K363Q), rat P2X5 (WT, S365K-E366Y or S365K-E366Y-E374K) or human P2X5 (kind gift from Dr Alan North). Oocytes were then incubated in Barth’s solution containing 1.8 mM CaCl₂ at 19°C for 24 to 72 h before electrophysiological recordings. Two-electrode voltage-clamp recordings (Vhold = −60 mV for P2X4, −120 mV for P2X5) were performed using glass pipettes (1–3 MΩ) filled with 3 M KCl solution. The external Ringer’s solution, pH 7.4, contained (in mM): 115 NaCl, 5 NaOH, 2.5 KCl, 1.8 CaCl₂, and 10 HEPES. Membrane currents were recorded using a Warner OC-725B amplifier (Warner Instruments) and digitized at 1 kHz. For PIP₆-depletion experiments, oocytes were incubated in 10 μM wortmannin for 1–2 hours prior to recording. Each series of recordings consisted of three successive applications of ATP (10 or 100 μM), with a 4-minute wash in Ringer’s solution between each application.

Figure 5. P2X C-terminal peptides compete with P2X channels for binding to intracellular PIP₆. A) Representative traces of patch-clamp recordings showing that intracellular injection of the P2X4 C-terminal (CT) peptide leads to a rundown of the P2X4 current in HEK293 cells by competing for intracellular PIP₆. B) Intracellular injection of the P2X5-CT peptide does not affect the P2X4 current amplitude of injection of peptides from the P2X4 WT, P2X4 K362Q-K363Q (2M), P2X5 WT or P2X5 S365K-E366Y-E374K (3M) C-terminus. D) Competition for PIP₆ binding from P2X4-CT or P2X5-3M-CT peptide injection leads to a slower desensitization of the P2X4 current. Values were normalized to the initial recording value obtained immediately after whole-cell configuration was obtained (n = 4–5; *, p<0.05; **, p<0.01; ***; p<0.001, each group compared to control). doi:10.1371/journal.pone.0040595.g005
Whole-cell Patch-clamp Recordings on HEK293 Cells

HEK293 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium and 10% heat-inactivated fetal bovine serum (Invitrogen) containing penicillin and streptomycin supplemented with G-418 (250 μg/ml). The cells were transiently co-transfected with pEGFP and pCDNA3-rP2X4 using Polyfect (Qiagen) according to the manufacturer’s instructions. Transfected cells were used for electrophysiological recordings 48 h after transfection. Whole-cell recordings (V_hold = −60 mV) were performed using pipettes filled with internal solution, pH 7.2, containing (in mM): 120 K-gluconate, 1 MgCl2, 5 EGTA and 10 HEPES. The recording solution, pH 7.4, comprised (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES and 10 glucose. GST-fusion peptides (GST control, P2X4-CT, P2X4 K362Q-K363Q-CT, P2X5-CT or P2X5 S365K-E366Y-E374K-CT) were dissolved in the internal pipette solution to a 1 μM concentration. Membrane currents were recorded using an Axopatch 200 amplifier and digitized at 500 Hz with a Digidata 1330 interface (Axon Instruments). Agonists were dissolved in recording solution and applied using a SF-77B fast perfusion system (Warner Instruments) at a rate of 1 ml/min.

Site-directed Mutagenesis

Point mutations on pcDNA3-rP2X4 (K362Q-K363Q) and pcDNA3-rP2X5 (S365K-E366Y-E374K) were introduced using the QuikChange mutagenesis method (Stratagene).

Phospholipid-binding Assay

Oligonucleotide-based sequences coding for P2X C-terminal sequences (P2X1: L352-E378, P2X4: C360-V375, P2X5: L361-V376, P2X7: P376-I391, P376-P396, A378-E393) were subcloned into the pGEX-2T vector for the production of GST fusion proteins (1 μg/ml). The cells were transiently co-transfected with pEGFP and pCDNA3-rP2X4 using Polyfect (Qiagen) containing penicillin and streptomycin supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) containing penicillin and streptomycin supplemented with 10% heat-inactivated fetal bovine serum. The cells were transiently co-transfected with pEGFP and pCDNA3-rP2X4 using Polyfect (Qiagen) containing penicillin and streptomycin supplemented with 10% heat-inactivated fetal bovine serum. The cells were transiently co-transfected with pEGFP and pCDNA3-rP2X4 using Polyfect (Qiagen) containing penicillin and streptomycin supplemented with 10% heat-inactivated fetal bovine serum.
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