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DIRECT MODULATION OF P2X₁ RECEPTOR-CHANNELS BY THE LIPID PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE

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Abstract

The P2X₁ receptor-channels activated by extracellular ATP contribute to the neurogenic component of smooth muscle contraction in vascular beds and genito-urinary tracts of rodents and humans. In the present study, we investigated the interactions of plasma membrane phosphoinositides with P2X₁ ATP receptors and their physiological consequences. In an isolated rat mesenteric artery preparation, we observed a strong inhibition of P2X₁-mediated constrictive responses by depletion of PI(4,5)P₂ with the PI4-kinase inhibitor wortmannin. Using the *Xenopus* oocyte expression system, we provided electrophysiological evidence that lowering PI(4,5)P₂ levels with wortmannin significantly decreases P2X₁ currents amplitude and recovery. Previously reported modulation of recovery of desensitized P2X₁ currents by phospholipase C-coupled 5-HT_{2A} metabotropic receptors was also found wortmannin-sensitive. Treatment with wortmannin alters the kinetics of P2X₁ activation and inactivation without changing its sensitivity to ATP. The functional impact of wortmannin on P2X₁ currents could be reversed by addition of intracellular PI(4,5)P₂, but not PI(3,4,5)P₃, and direct application of PI(4,5)P₂ to excised inside-out macropatches rescued P2X₁ currents from rundown. We showed that the proximal region of the intracellular C-terminus of P2X₁ subunit directly binds to PI(4,5)P₂ and other anionic phospholipids, and we identified the basic residue K364 as a critical determinant for phospholipid binding and sensitivity to wortmannin. Overall, these results indicate that PI(4,5)P₂ plays a key role in the expression of full native and heterologous P2X₁ function by regulating the amplitude, recovery and kinetics of ionotropic ATP responses through direct receptor-lipid interactions.

P2X receptors are nonselective cation channels gated by extracellular ATP. P2X subunits (P2X₁₋₇) assemble to form either homo- or hetero-trimeric channels, giving rise to a wide range of current phenotypes (North, 2002). Among P2X receptors, the P2X₁ subtype shows high sensitivity to the agonist α,β -methylene ATP (α,β -meATP), fast inactivation kinetics and high expression in smooth muscle cells lining the urinary bladder, arteries, and vas deferens, where it regulates contractility (Sneddon, 2000; Burnstock, 2007). The activity of P2X₁ receptor-channels can be modulated by phospholipase C-mediated signaling events (Roberts et al., 2006). For instance, recovery of P2X₁ responses can be accelerated by activation of α_1 -adrenergic receptors in the vas deferens of guinea pig (Smith and Burnstock, 2004) or by activation of 5-HT_{2A} serotonin receptors in the rat tail artery (Ase et al., 2005). In recombinant studies, P2X₁ receptors currents have been shown to be potentiated by stimulation of co-expressed Gq-coupled mGluR₁, P2Y_{1/2} and 5-HT_{2A} receptors (Vial et al.,

2004; Ase et al., 2005). By using cholesterol-depleting agents, Vial and Evans (2005) reported reduced α,β -meATP-induced contractions of the rat tail artery and decreased P2X₁-mediated currents in transfected HEK293 cells, suggesting that lipids regulate P2X₁ receptor signaling. Phosphoinositides have recently been shown to modulate P2X₂ and P2X₇ receptors (Fujiwara and Kubo, 2006; Zhao et al., 2007). Phosphoinositides are produced by specific lipid kinases through phosphorylation of phosphatidylinositol (PI) at the 3', 4' or 5' position of the inositol ring. Phosphoinositides exert their regulatory role either indirectly as precursors of the phospholipase C-generated second messengers inositol 1,4,5-trisphosphate and diacylglycerol, or directly by interacting with proteins and thus modulating their localization, conformation, and activity. PI(4,5)P₂, one of the most abundant phosphoinositide in the cell membrane, regulates many cellular events such as cytoskeleton remodeling, vesicle transport and ion channels or transporters function (Suh and Hille, 2005; Gamper and Shapiro, 2007).

In the present study, we aimed to explore the interaction of PI(4,5)P₂ with native and recombinant P2X₁ receptor-channels and its physiological consequences. We report here, using the isolated rat mesenteric artery preparation, a strong modulation of P2X₁-mediated vascular reactivity by wortmannin-induced PI(4,5)P₂ depletion. Using two-electrode voltage clamp and inside-out patch recordings in the *Xenopus* oocyte expression system, we also provide functional evidence that PI(4,5)P₂ modulates P2X₁ current amplitude, recovery, speed of activation and inactivation, and is required for the 5-HT_{2A}-mediated potentiation of P2X₁ currents. Finally, using an in vitro lipid binding assay and mutagenesis, we show that the proximal C-terminal region of P2X₁ subunit, containing the critical basic residue K364, directly interacts with several anionic phospholipids including PI(4,5)P₂.

Materials and Methods

Reactivity of Isolated Rat Mesenteric Arteries

All procedures were as described previously (Elhusseiny and Hamel, 2000; Tong et al., 2005). Briefly, rats were sacrificed by cervical dislocation and the mesenteric arteries isolated and collected in cold Krebs solution (4°C, pH 7.4) containing the following (in mM): 118 NaCl, 4.5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 KH₂PO₄, 25 NaHCO₃, and 11 glucose. Arterial segments (about 2 mm long) were transferred to a small superfusion chamber (5 ml, Living System), cannulated on a glass micropipette (80 μ m diameter) at one end, sealed to another glass micropipette at the other end, and filled with oxygenated (5% CO₂ and 95% O₂) Krebs solution (37°C, pH 7.4). Intraluminal pressure was maintained at 60 mmHg, vessels were superfused with Krebs solution and allowed to stabilize and acquire basal tone. On-line measurements of intraluminal diameter were performed using a closed-circuit video system coupled with a video caliper. Direct abluminal application of α,β -meATP (10 μ M) was performed on vessels pre-treated or not with the PI3/4-kinases inhibitor wortmannin (100 nM or 1 μ M). In the wortmannin experiments, vessels were rapidly washed with fresh Krebs solution before application of α,β -meATP.

Expression of Recombinant P2X₁ Receptors in *Xenopus* Oocytes

Ovary lobes were surgically removed from *Xenopus laevis* frogs deeply anesthetized by immersion in 0.2% Tricaine (Sigma-Aldrich, St. Louis, MO). After treatment with type I collagenase in calcium-free OR2 solution for 2 h at room temperature, stage V–VI oocytes were manually defolliculated. Wild-type or mutant rat P2X₁ subunit and rat 5-HT_{2A} receptor cRNAs were transcribed in vitro using the mMessage mMachine kit (Ambion, Austin, TX) from pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA) and then microinjected into the cytoplasm of oocytes. For expression of P2X₁ alone 25 ng of cRNA were injected in each oocyte. In co-expression experiments, 25 ng of P2X₁ and 25 ng of 5-

HT_{2A} cRNA were injected. Oocytes were kept at 19°C in Barth solution containing 50 µg/ml of gentamycin and 1.8 mM CaCl₂ for 48 h before recording.

Electrophysiology

Two-electrode voltage clamp recordings in *Xenopus* oocytes expressing P2X₁ receptors were performed at room temperature using an OC-725C amplifier (Warner Instruments, Hamden, CT) and glass pipettes (1–3 MΩ) filled with 3 M KCl. Oocytes were placed in a 500-µl recording chamber and were perfused at a flow rate of 10–12 ml/min with Ringer solution, pH 7.4, containing 115 mM NaCl, 5 mM NaOH, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES. Oocytes were held at –60 mV during recording. Unless specified, stimulations with 10 µM ATP (Sigma-Aldrich) dissolved in Ringer solution were applied consecutively at 5-min intervals and P2X₁ responses were stable after the second application of ATP. When wortmannin was used, oocytes were incubated for 2 hours in Barth solution containing 100 nM or 35 µM wortmannin, or vehicle (0.3% DMSO), prior to recording. For experiments involving co-expression of 5-HT_{2A} receptors, 5-HT (1 µM) was applied in the bath for 5 min between the third and fourth ATP application. Potentiation index was defined as the ratio of the fourth over the third ATP-evoked P2X₁ peak current. The phospholipids diC8-PI(4,5)P₂ or diC8-PI(3,4,5)P₃ (Echelon Biosciences) were injected (20 nl, 10 mM) in the cytoplasm of oocytes 30 min prior to final recording. For all the calculations of final drug concentration, we estimated the oocyte cell volume at 1 µl.

Inside-out macropatch recordings in *Xenopus* oocytes expressing P2X₁ channels were performed using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). PClamp 9 was used for data acquisition and analysis. The sampling rate was 2 kHz. A ramp protocol from –100 mV to +100 mV was applied once per second, and current amplitudes were acquired at –80 mV. Electrodes with resistance of 0.5–1.0 MΩ were used. The internal (bath) solution contained (in mM): 96 KCl, 0.5 EGTA, 10 HEPES, pH 7.4. The external (pipet) solution contained also 1.8 mM CaCl₂, as well as 20 µM ATP. Solutions were applied through a gravity-driven perfusion system. For each experiment, a minimum of two batches of oocytes were tested.

Lipid Strip Binding Assay

Annealed oligonucleotides coding for a 16 amino acid sequence proximal to the C-terminus of rat P2X₁ (I₃₅₆–A₃₇₁) flanked by BamHI-EcoRI ends were subcloned into pGEX-2T vector for production of wild-type or mutant GST-P2X₁ fusion proteins, before purification on glutathione-sepharose resin. Lipid binding analysis of GST-P2X₁ fusion proteins was conducted in vitro using identical amounts of phosphoinositides spotted on nitrocellulose membranes (PIP Strips™, Echelon Biosciences Inc) and the GST-N-terminus of P2X₁ or GST alone were used as negative controls. The membranes were blocked with TBS+T solution supplemented with 3% BSA for 1 hour at room temperature, then incubated overnight with 5 µg/ml of GST-fusion proteins in TBS+T with 3% BSA. The membranes were washed with TBS+T six times. Mouse anti-GST antibody (1:1000) was then added in TBS+T, 3% BSA solution for 1 hour at room temperature. Repeated washes with TBS+T were followed by 1 hour incubation with the secondary antibody HRP-labelled goat anti-mouse (1:5000) in TBS+T, 3% BSA at room temperature for detection of GST-P2X₁ fusion protein binding with enhanced chemiluminescence.

Statistical Analysis

All the values are expressed as mean ± S.E.M. Data were analyzed using Student's *t* test or one-way analysis followed by Newman-Keuls multiple comparison tests, with *p* < 0.05 considered significant.

Results

PI(4,5)P₂ depletion decreases P2X₁-mediated constrictive responses in rat mesenteric artery

Using the model of isolated rat mesenteric artery, we observed that initial applications of the selective P2X agonist α,β -meATP (10 μ M) to vessel segments led to strong muscle constriction responses ($69 \pm 2\%$ of maximal constriction induced by KCl, $n = 6$, Fig. 1). Micromolar concentrations of wortmannin, expected to inhibit both PI3-kinases and PI4-kinases, were used to test the impact of lower levels of phosphoinositides on native P2X₁-mediated constrictive responses. When the mesenteric vessels were incubated with 1 μ M wortmannin for 1 hour, α,β -meATP-mediated constriction was significantly decreased to $28 \pm 16\%$ ($n = 5$) (Fig. 1B). However, no significant changes were measured at a lower (100 nM) concentration of wortmannin that inhibits PI3-kinases only ($67 \pm 9\%$ of α,β -meATP-mediated constriction, $n = 3$, Fig. 1B).

PI(4,5)P₂ depletion reduces P2X₁ current amplitude and recovery in *Xenopus* oocytes without changing its sensitivity to ATP

We studied the effects of altering PI(4,5)P₂ levels on ATP-evoked currents in *Xenopus* oocytes expressing recombinant P2X₁ receptor channels. The oocytes were pretreated with vehicle or wortmannin at high (35 μ M) or low (100 nM) concentration for 2 hours before they were stimulated with 10 μ M ATP ($EC_{50} = 1 \mu$ M) for 10 seconds. We observed that high but not low concentration of wortmannin significantly reduced ATP-induced current amplitudes ($45.1 \pm 5.5\%$ of control, $n = 23$) as illustrated by the representative traces and quantitative results in Figure 2A and 2B respectively. Desensitization of P2X₁ responses following repeated applications of ATP was significantly stronger in oocytes incubated with 35 μ M wortmannin: 2nd response = $21 \pm 3\%$, of initial response, 3rd response = $14 \pm 2\%$ ($n = 14$) compared with 2nd response = $34 \pm 4\%$, 3rd response = $29 \pm 3\%$ ($n = 18$) for vehicle-treated oocytes (Fig. 2C). The inhibitory effect of wortmannin was found to depend on the concentration of ATP (Fig. 2D). When ATP ($EC_{50} = 1 \mu$ M) was applied at 0.1 μ M, the treatment with 35 μ M wortmannin had no effect on the current amplitude ($3.03 \pm 0.5 \mu$ A in vehicle-treated oocytes, $n = 6$, compared to $3.05 \pm 0.3 \mu$ A in wortmannin-treated oocytes, $n = 9$). A maximal inhibitory effect was reached at 100 μ M ATP ($27 \pm 5\%$ of control, $n = 9$). These results indicated that depletion of phosphoinositides with 35 μ M wortmannin blunted both the maximal response and the recovery of P2X₁ receptors without decreasing their sensitivity to ATP.

Potentiation of P2X₁ responses via 5-HT_{2A} activation is blocked by wortmannin induced PI(4,5)P₂ depletion

Oocytes coexpressing P2X₁ and Gq-coupled 5-HT_{2A} receptors were exposed to Ringer solution or 1 μ M 5-HT for 5 min between the third and fourth ATP applications. Under these conditions, 5-HT transiently elicited inward currents (Fig. 3A,C), sometimes oscillatory in nature, indicating secondary activation of endogenous Ca²⁺-dependent chloride currents. Application of 5-HT significantly potentiated P2X₁ current responses, as previously reported (Ase et al., 2005) and illustrated in Figure 3A (see also Fig. 3D for quantitative results). However, 2 hours of preincubation with 35 μ M wortmannin blocked the accelerated recovery of P2X₁ currents following 5-HT_{2A} activation (Fig. 3B,D). Lower concentration of wortmannin (100 nM) did not have any significant effect on 5-HT-induced modulation of P2X₁ currents (Fig. 3C,D).

Rescue of P2X₁ current responses and kinetics by PI(4,5)P₂

To study the effect of PI(4,5)P₂ on P2X₁ receptor-channels expressed in oocytes, we injected diC8-PI(4,5)P₂ (200 μM) in the cytoplasm 30 min before recording. Under basal conditions, ATP-evoked P2X₁ current amplitudes and kinetics were not affected by the addition of diC8-PI(4,5)P₂ (data not shown). However, in oocytes preincubated with 35 μM wortmannin, diC8-PI(4,5)P₂ addition led to a significant rescue of P2X₁ current amplitudes: $2.8 \pm 0.5 \mu\text{A}$ (n= 8) compared to $1.0 \pm 0.2 \mu\text{A}$ in PBS-injected oocytes (n= 14) (Fig. 4A,B). Thus, whereas addition of diC8-PI(4,5)P₂ did not seem to increase ATP-evoked P2X₁ currents under basal conditions, it did so when endogenous levels of PI(4,5)P₂ were previously depleted by wortmannin treatment. In contrast, diC8-PI(3,4,5)P₃ (200 μM) injections did not have any rescuing effect on the current amplitudes of P2X₁ receptors following wortmannin treatment (Fig. 4B).

In addition, we found that PI(4,5)P₂ levels have a significant impact on the kinetics of P2X₁ current responses. Thus, as illustrated by the current traces in Figure 5A, treatment with high concentration of wortmannin clearly slowed down both the 10–90% rise time of the activation phase and the inactivation time (one-exponential fit) of P2X₁ current responses. The 10–90% rise time was $0.17 \pm 0.01 \text{ s}$ (n= 9) in control conditions and $0.25 \pm 0.03 \text{ s}$ (n= 9) after wortmannin. The time constant of inactivation was $0.92 \pm 0.07 \text{ s}$ (n= 9) in control conditions and $1.42 \pm 0.14 \text{ s}$ (n= 9) after wortmannin. We observed that injection of PI(4,5)P₂ completely restored the kinetic parameters of P2X₁ responses to their control levels (Fig. 5B,C).

PI(4,5)P₂ activates P2X₁ current responses in excised macropatches

To test direct effects of phosphoinositides on heterologously expressed P2X₁ receptors, we applied diC8-PI(4,5)P₂ to inside-out macropatch membranes from *Xenopus* oocytes. Upon patch excision, a fast rundown of the current was observed. Application of 100 μM polylysine, which sequesters PI(4,5)P₂, stabilized the P2X₁ currents to basal levels (Fig. 6A). Subsequent application of 5 μM diC8-PI(4,5)P₂ strongly activated P2X₁ currents (Fig. 6A). A quantitative analysis shows significantly larger amplitude (~ 2-fold) for P2X₁ currents in the presence of diC8-PI(4,5)P₂, compared to rundown baseline levels (Fig. 6B).

The single lysine K364 in the C-terminus of P2X₁ subunit is a critical determinant for binding phosphoinositides

Because phosphoinositides are anionic, we tested for potential interactions of these lipids with positively charged residues on the intracellular domains of the P2X₁ subunits by performing a lipid strip assay. The proximal region of the C-terminal domain displays a cluster of basic amino acids (Fig. 7A) that are potentially involved in the interaction with phosphoinositides, as recently reported for the P2X₂ receptor (Fujiwara and Kubo, 2006; Zhao et al., 2007). No specific binding was observed when a GST-P2X₁ fusion protein expressing the N-terminal peptide F₁₂-V₂₉ was tested (Fig. 7B). However the GST-P2X₁ fusion protein containing the C-terminal peptide I₃₅₆-A₃₇₁ selectively bound to several phospholipids including PI(3)P, PI(4)P, PI(5)P, PI(3,5)P₂, PI(4,5)P₂, PI(3,4,5)P₃, PS, and to a lesser extent, PI(3,4)P₂ and PA, as illustrated in Fig. 7B. We investigated the contribution of each lysine or arginine residue in the C-terminal peptide by replacing them with the neutral residue glutamine. Only one substitution, K364Q, led to the complete suppression of binding (Fig. 7B) therefore the lysine K364 is a critical determinant for interaction with phosphoinositides. As shown in Figure 7C, ATP-evoked current amplitude was smaller in oocytes expressing the mutant receptor P2X₁ K364Q ($4.94 \pm 0.69 \mu\text{A}$, n= 10) compared to wild-type receptor ($8.27 \pm 0.99 \mu\text{A}$, n= 10). Moreover, P2X₁ K364Q receptors showed higher sensitivity to depletion of PI(4,5)P₂ induced by 35 μM wortmannin ($13.70 \pm 1.80 \%$

of control response, $n= 19$) than wild-type receptors (44.88 ± 10.13 % of control response, $n= 9$), confirming a decrease in PI(4,5)P₂ binding affinity (Fig. 7D).

Discussion

The homomeric P2X₁ purinoceptor is an ATP-gated receptor-channel mainly expressed in smooth muscles of the genito-urinary tract and vessels, where it mediates neurogenic contraction (Valera et al., 1994; Hansen et al., 1998). This purinergic component of smooth muscle contraction is present in rodents (Mulryan et al., 2000) as well as in humans (Banks et al., 2006). In the present study, we demonstrated the modulation of P2X₁ responses by phosphoinositides and its physiological relevance since α , β -meATP-evoked P2X₁-mediated constrictions of the rat mesenteric artery are sensitive to the pharmacological depletion of PI(4,5)P₂. We were able to reconstitute in the *Xenopus* oocyte expression system the modulation of P2X₁-mediated responses by blocking the critical lipid kinases involved in phosphoinositides synthesis, thus indicating that ubiquitous intracellular mechanisms are involved. Two-electrode voltage-clamp recordings showed decreased ATP-evoked P2X₁ current responses with high (μ M) concentration of wortmannin, required to block both PI4-kinases and PI3-kinases (Nakanishi et al., 1995), whereas low (nM) concentrations of wortmannin, known to block PI3-kinases only, did not have any effect. The inhibitory effect of wortmannin is not due to a decrease in sensitivity of P2X₁ receptors to ATP because 1) the inhibition was unsurmountable at supramaximal concentrations of ATP, 2) maximal current amplitude was reached at 10 μ M ATP after wortmannin treatment as in control conditions, and 3) wortmannin had no effect on P2X₁ currents induced by 0.1 μ M ATP. We can conclude that ATP becomes a partial agonist for P2X₁ when PI(4,5)P₂ levels are depleted. In other words, minimal PI(4,5)P₂ levels are required for the expression of a full P2X₁ response to ATP. Recovery of P2X₁ responses was also found sensitive to treatment with high concentration of wortmannin, suggesting that desensitization of P2X₁ receptor-channels is regulated by PI(4,5)P₂.

We and others have reported the potentiation of P2X₁ responses by activation of Gq-coupled receptors (Vial et al., 2004; Ase et al., 2005) so we tested the impact of wortmannin on the modulation of P2X₁ responses by Gq-coupled 5-HT_{2A} receptors. The lack of potentiation of P2X₁ responses after blockade of PI4-kinases by 35 μ M wortmannin, as well as the absence of effect of PI3-kinases blockade by 100 nM wortmannin, supported a critical role for PI(4,5)P₂ as PLC substrate in the crosstalk between 5-HT_{2A} and P2X₁ receptors. The effectiveness of the blockade of PI4-kinases and PI(4,5)P₂ synthesis was confirmed by the suppression of IP₃-induced Ca²⁺-dependent chloride currents, triggered by 5-HT, after treatment with 35 μ M wortmannin. Stimulation of the PLC-coupled platelet-derived growth-factor tyrosine kinase receptor has been shown to decrease PI(4,5)P₂-sensitive P2X₇ currents (Zhao et al., 2007) however we did not observe any decrease of P2X₁ responses after 5-HT_{2A} stimulation. This suggests that a transient stimulation of Gq-coupled receptors does not significantly deplete the steady-state levels of PI(4,5)P₂ available at the plasma membrane, in agreement with what is known about the high turnover rate of this phospholipid (Hilgemann, 2007).

The specific involvement of PI(4,5)P₂ in modulating P2X₁ channels responses is directly supported by the fact that addition of PI(4,5)P₂, and not PI(3,4,5)P₃, significantly rescued the amplitude of P2X₁ currents after blockade of PI4-kinases with 35 μ M wortmannin. Decreased levels of phosphoinositides not only affected the amplitude of P2X₁ currents but also their kinetics, as wortmannin clearly slowed down their 10–90% rise time and increased their time constant of inactivation. Addition of intracellular PI(4,5)P₂ after treatment with wortmannin normalized the kinetics of the P2X₁ currents back to their control values. Under basal conditions however, increasing PI(4,5)P₂ levels by direct injection into the cytoplasm

did not affect neither basal P2X₁ current amplitudes nor activation/inactivation kinetics. Thus, at least in oocytes, endogenous PI(4,5)P₂ levels saturate the P2X₁ receptor-channels expressed at the cell surface.

Our results using inside/out macropatches excised from P2X₁-expressing oocytes provided evidence for the sensitivity of P2X₁ receptor-channels to direct application of PI(4,5)P₂. P2X₁ shows little activity upon patch excision due to a rapid rundown resulting from receptor desensitization and dephosphorylation of PI(4,5)P₂ by lipid phosphatases (Huang et al., 1998; Zhang et al., 1999). Direct regulation of channel activity by phosphoinositides in excised patches has been reported for several types of ion channels, including the prototypical Kir and KCNQ channels also modulated by PI(4,5)P₂ (Lopes et al., 2002; Suh and Hille, 2002, 2005; Zhang et al., 2003). Application of exogenous PI(4,5)P₂ on the intracellular side of the patch rescued P2X₁ currents from rundown, indicating the requirement of PI(4,5)P₂ for full P2X₁ function and arguing against trafficking to the surface as the mechanism for PI(4,5)P₂-induced potentiation of P2X₁ currents.

Searching for phospholipid-binding motifs in the intracellular sequences of P2X₁ subunits, we confirmed the direct binding of PI(4,5)P₂ to the carboxy-terminal domain I₃₅₆-A₃₇₁. This domain proximal to the second transmembrane domain, in a position favorable for interactions with the anionic head groups of phospholipids according to the transmembrane topology of P2X subunits, contains a high density of basic residues (5 lysines and 1 arginine) and we have found that the single lysine K364 plays a critical role in these interactions. The mutant receptor P2X₁ K364Q remains functional but with a lower binding affinity for PI(4,5)P₂ indicated by a higher sensitivity to wortmannin-induced depletion, analogously to mutants of other PI(4,5)P₂-sensitive channels (Rohàcs et al., 2005). The fact that the K364Q mutation in the functional P2X₁ receptor-channel did not lead to complete loss of PI(4,5)P₂ binding and insensitivity to wortmannin suggests that other determinants contribute to phosphoinositides binding. Interestingly, the C-terminal sequence of P2X₁ also showed strong affinity for other anionic phospholipids. PI(4,5)P₂ and PI(4)P are by far the most abundant phosphoinositides in the plasma membrane however the dependence of ion channel activity on other phosphoinositides is well documented (Zhaiazarov et al., 2004; Pochynyuk et al., 2005; Srivastava et al. 2005). A similar but qualitatively distinct lipid-binding profile was recently reported for the homologous C-terminal sequence of P2X₂ (Fujiwara and Kubo, 2006). Therefore, this short domain appears to be of general importance for the regulation of P2X function by phosphoinositides.

In conclusion we have provided strong evidence that the phospholipid PI(4,5)P₂ is a physiological ligand of P2X₁ receptor-channels, and our results suggest a novel mode of regulation of ionotropic P2X₁ responses by the various metabotropic pathways linked to phosphoinositides metabolism.

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Abbreviations

α,β-meATP	α,β -methylene ATP
PI	phosphatidylinositol

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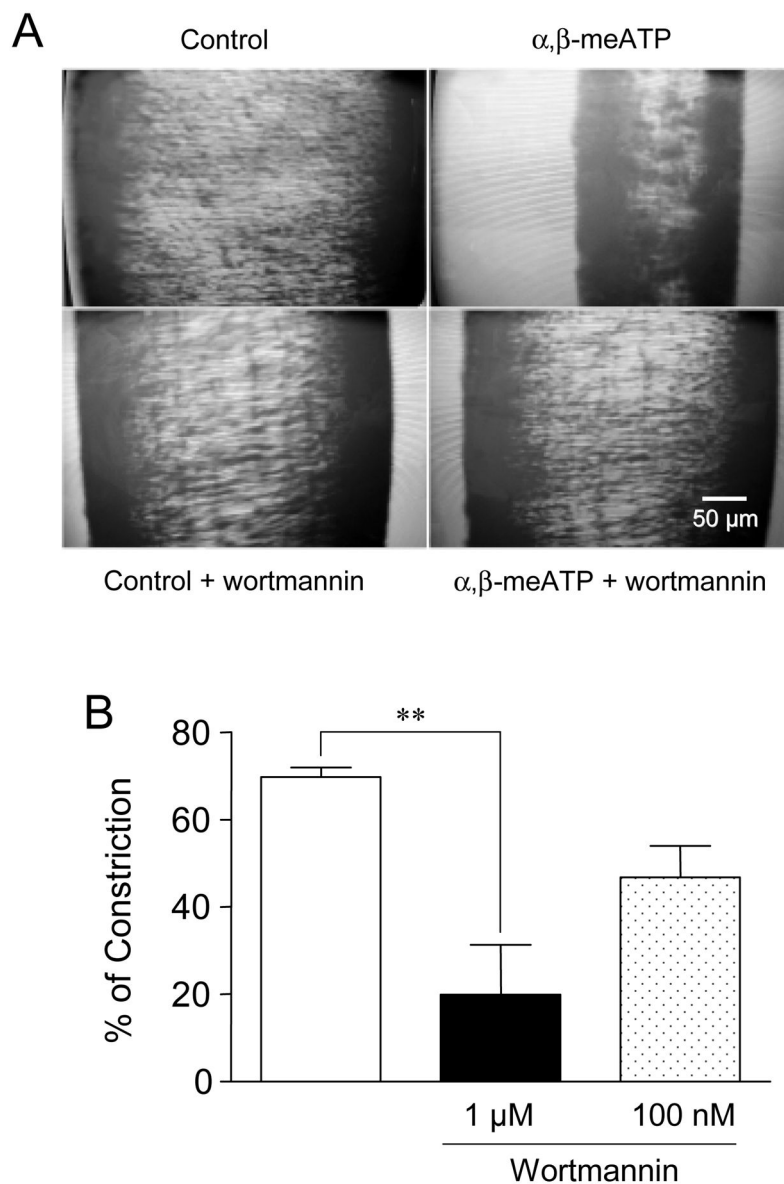


Figure 1. P2X₁ constrictive responses are sensitive to wortmannin

(A) Representative video recordings of changes in rat mesenteric artery diameter. *Control*: vessel diameter measured at baseline tone. *α,β -meATP*: same vessel one minute after application of α,β -meATP (10 μ M). *Control + wortmannin*: vessel diameter measured at baseline tone 30 min after application of wortmannin (1 μ M). *α,β -meATP + wortmannin*: same vessel, 1 minute after application of α,β -meATP (10 μ M). (B) Constriction responses elicited by application of the P2X agonist α,β -meATP (10 μ M) on rat mesenteric artery and their partial blockade after treatment with 1 μ M wortmannin (n= 3–6, ** $p < 0.01$).

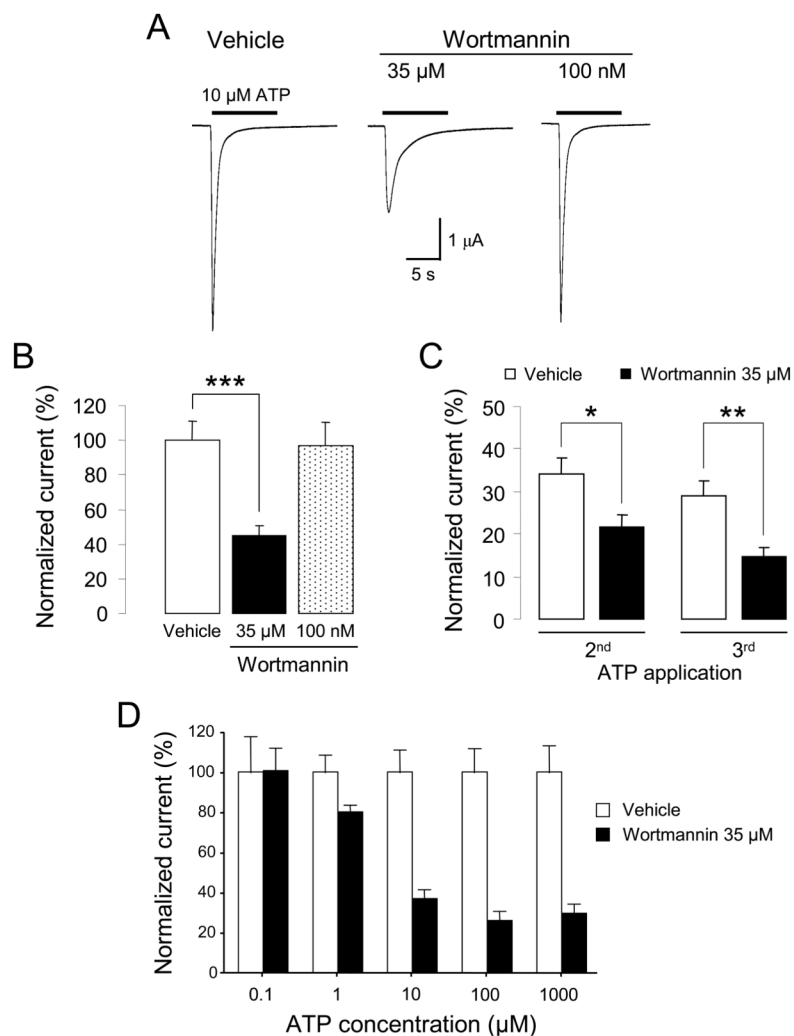


Figure 2. Inhibitory effect of wortmannin on recombinant P2X₁ current responses (A) Representative ATP-evoked inward currents in *Xenopus* oocytes expressing P2X₁ receptors. High (35 μM) but not low (100 nM) concentration of wortmannin decreases P2X₁ peak current. (B) Quantitative results (n= 9–17, *** $p < 0.001$). (C) Treatment with 35 μM wortmannin decreases recovery of P2X₁ currents following consecutive applications of ATP (n= 14–18, * $p < 0.05$; ** $p < 0.01$). (D) The inhibitory effect of wortmannin is agonist concentration-dependent (n= 6–17).

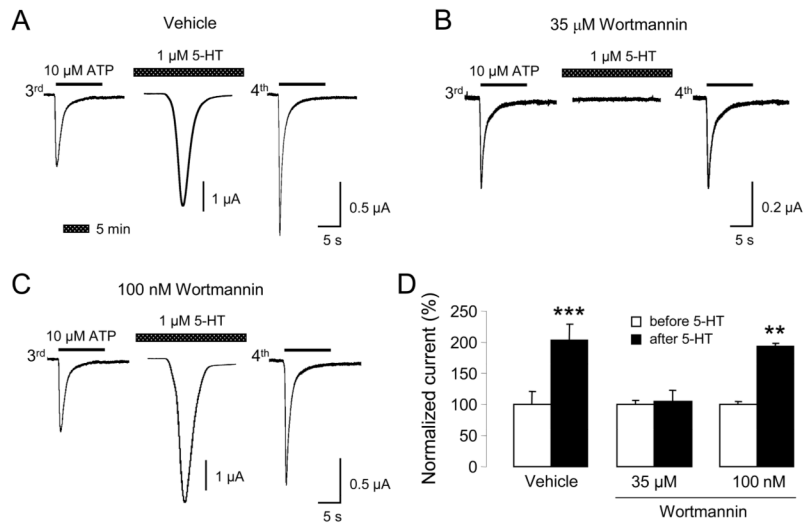


Figure 3. Wortmannin blocks 5-HT_{2A}-mediated recovery of P2X₁ currents

(A) Representative ATP-evoked (3rd and 4th application) and 5-HT-evoked inward currents from oocytes coexpressing P2X₁ and 5-HT_{2A} receptors. A 5 min application of 5-HT accelerates recovery of P2X₁ current amplitudes. (B) Traces show blockade of accelerated recovery of P2X₁ current responses by preincubation with 35 μM wortmannin (inhibition of PI3- and PI4-kinases). Note that high wortmannin concentration also suppresses 5-HT-evoked Ca²⁺-activated chloride currents. (C) Traces show absence of effect when low concentration of wortmannin (PI3-kinases inhibition only) is used. (D) Quantification of recovery of P2X₁ current responses, normalized to the responses before 5-HT application (n= 5–7, ** *p* < 0.01; *** *p* < 0.001).

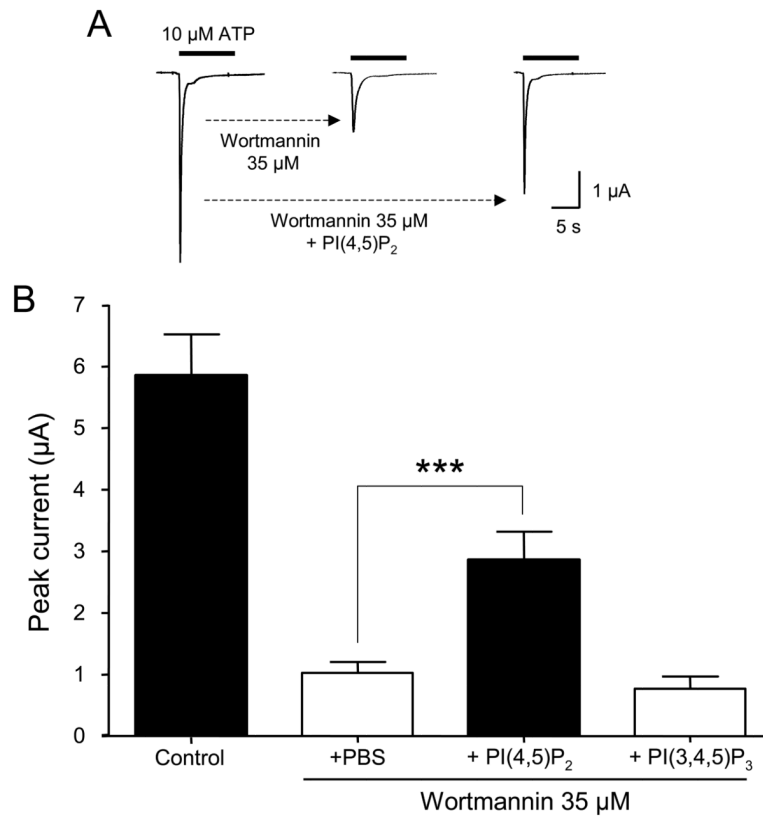


Figure 4. PI(4,5)P₂ specifically rescues P2X₁ current amplitude following wortmannin-mediated blockade

(A) Representative traces showing ATP-evoked inward current (left), its partial blockade by wortmannin (middle), and its rescue (right) by injection of diC8-PI(4,5)P₂ into oocytes expressing P2X₁ receptors. (B) Quantitative summary of the results, showing the lack of rescue when diC8-PI(3,4,5)P₃ is injected (n= 9, *** $p < 0.001$).

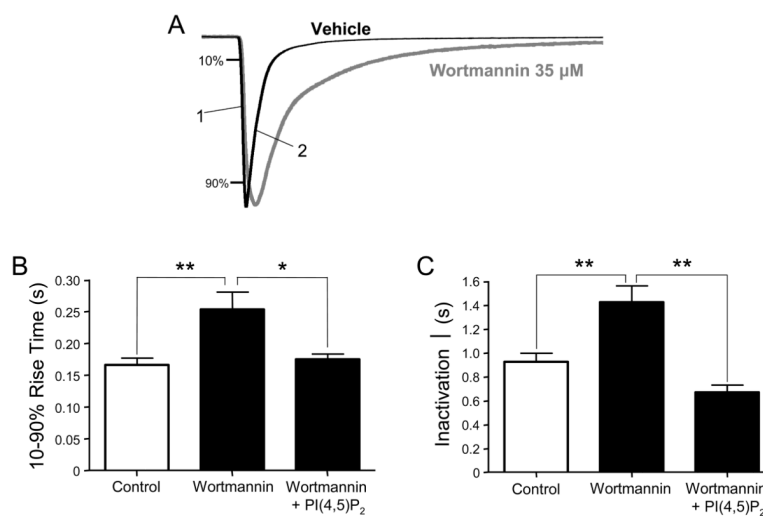


Figure 5. PI(4,5)P₂ depletion slows down activation and inactivation of P2X₁ currents
 (A) Representative traces showing slower activation (1) and inactivation (2) of ATP-evoked P2X₁ inward current after wortmannin treatment. (B) Quantitative results show the complete rescue of kinetic parameters of P2X₁ currents to normal levels by PI(4,5)P₂ delivery into oocytes (n= 9, * *p* < 0.05; ** *p* < 0.01).

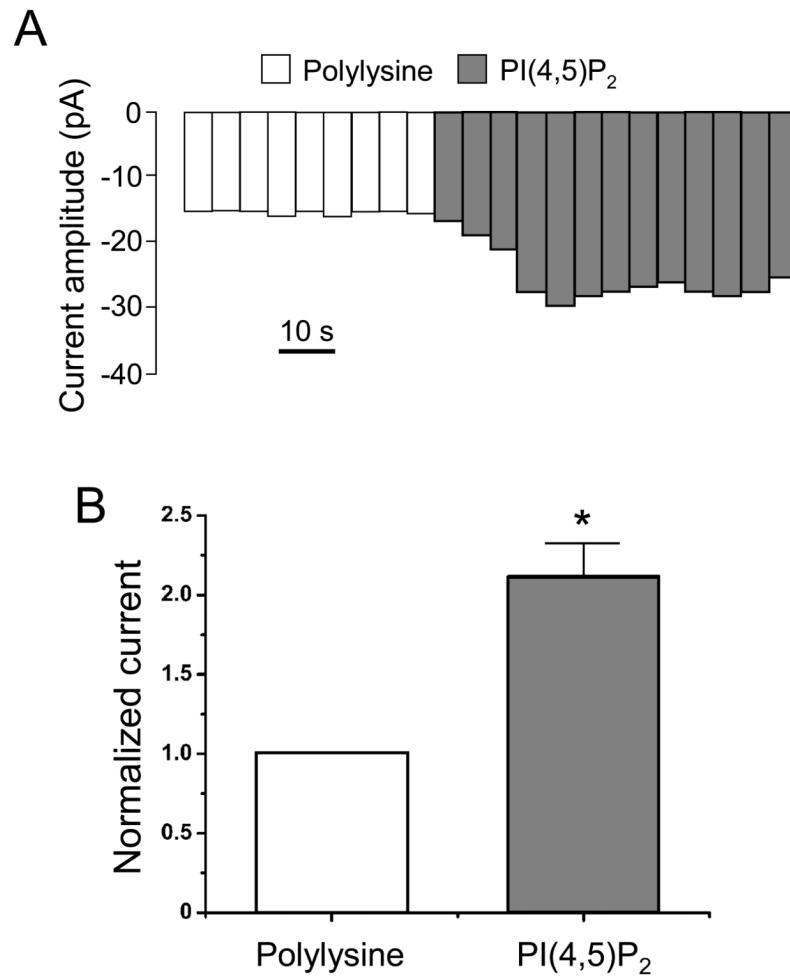


Figure 6. PI(4,5)P₂-activated P2X currents in excised macropatches from *Xenopus* oocytes
 (A) Averaged current amplitudes during 5 s intervals at -80 mV. Currents were evoked by ramps from -100 mV to $+100$ mV. The internal pipette solution contained 20 μ M ATP. Polylysine is applied on the intracellular side at 100 μ M. (B) Quantitative results show rescue of P2X₁ currents from ATP-induced rundown by 5 μ M diC8- PI(4,5)P₂ application ($n=4-9$, * $p < 0.05$).

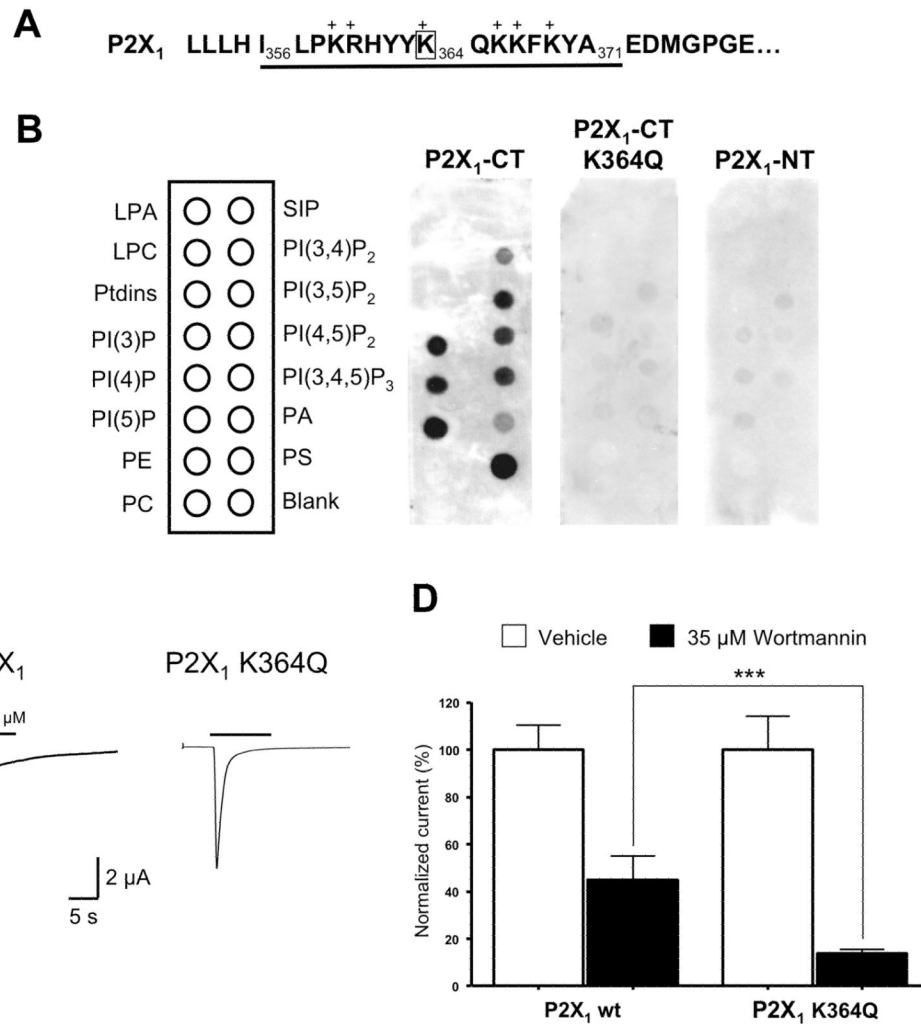


Figure 7. Lysine K364 in the proximal C-terminal domain of P2X₁ subunit is a critical determinant for phosphoinositides binding and sensitivity to wortmannin

(A) Sequence of the proximal C-terminal domain of rat P2X₁ subunit. (B) The GST construct containing the proximal C-terminal domain I₃₅₆-A₃₇₁ of the P2X₁ subunit binds to several anionic phosphoinositides, including PI(4,5)P₂. Binding is suppressed when the single lysine K364 is replaced with glutamine. No binding is detected when using the GST construct containing the N-terminal domain of P2X₁ (P2X₁-NT) as negative control. LPA: Lysophosphatidic Acid, LPC: Lysophosphocholine, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, SIP: Sphingosine-1-phosphate, PA: Phosphatidic Acid, PS: Phosphatidylserine. (C) Representative traces showing that the mutant receptor P2X₁ K364Q carries smaller ATP-evoked currents than the wild-type P2X₁ receptor (n = 10, * *p* < 0.05). (D) The mutant receptor P2X₁ K364Q is also more sensitive than the wild-type receptor to depletion of PI(4,5)P₂ induced by 35 μM wortmannin (n = 9–19, *** *p* < 0.001).