MOL
PHARM

MOLECULAR PHARMACOLOGY

Direct Modulation of P2X$_1$ Receptor-Channels by the Lipid Phosphatidylinositol 4,5-Bisphosphate

Louis-Philippe Bernier, Ariel R. Ase, Xinkang Tong, Edith Hamel, Dominique Blais,
Qi Zhao, Diomedes E. Logothetis, and Philippe Séguéla

Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada
(L.-P.B., A.R.A., X.T., E.H., D.B., P.S.); and Department of Structural and Chemical Biology, Mount Sinai School of Medicine,
New York University, New York, New York (Q.Z., D.E.L.)

ABSTRACT

The P2X$_1$ receptor-channels activated by extracellular ATP contribute to the neurogenic component of smooth muscle contraction in vascular beds and genitourinary tracts of rodents and humans. In the present study, we investigated the interactions of plasma membrane phosphoinositides with P2X$_1$ ATP receptors and their physiological consequences. In an isolated rat mesenteric artery preparation, we observed a strong inhibition of P2X$_1$-mediated constrictive responses by depletion of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] with the phosphatidylinositol 4-kinase inhibitor wortmannin. Using the *Xenopus laevis* oocyte expression system, we provided electrophysiological evidence that lowering PI(4,5)P$_2$ levels with wortmannin significantly decreases P2X$_1$ current amplitude and recovery. Previously reported modulation of recovery of desensitized P2X$_1$ currents by phospholipase C-coupled 5-hydroxytryptamine$_{2A}$ metabotropic receptors was also found to be wortmannin-

sensitive. Treatment with wortmannin alters the kinetics of P2X$_1$ activation and inactivation without changing its sensitivity to ATP. The functional impact of wortmannin on P2X$_1$ currents could be reversed by addition of intracellular PI(4,5)P$_2$, but not phosphatidylinositol 3,4,5-trisphosphate, and direct application of PI(4,5)P$_2$ to excised inside-out macropatches rescued P2X$_1$ currents from rundown. We showed that the proximal region of the intracellular C terminus of P2X$_1$ subunit directly binds to PI(4,5)P$_2$ and other anionic phospholipids, and we identified the basic residue Lys$_{364}$ as a critical determinant for phospholipid binding and sensitivity to wortmannin. Overall, these results indicate that PI(4,5)P$_2$ plays a key role in the expression of full native and heterologous P2X$_1$ 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 (P2X$_{1-7}$) assemble to form either homo- or heterotrimeric channels, giving rise to a wide range of current phenotypes (North, 2002). Among P2X receptors, the P2X$_1$ 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 P2X$_1$ receptor-channels can be modulated by

phospholipase C-mediated signaling events. For example, recovery of P2X$_1$ 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, P2X$_1$ receptor currents have been shown to be potentiated by stimulation of coexpressed G$_q$-coupled metabotropic glutamate receptor$_1$, 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 P2X$_1$-mediated currents in transfected human embryonic kidney 293 cells, suggesting that lipids regulate P2X$_1$ receptor signaling. Phosphoinositides have recently been shown to modulate P2X$_2$ and P2X$_7$ receptors (Fujiwara and Kubo, 2006; Zhao et al., 2007). Phosphoinositides are produced by specific lipid kinases through phosphor-

ABBREVIATIONS: α,β-meATP, α,β-methylene ATP; 5-HT, 5-hydroxytryptamine; PI, phosphatidylinositol; PI(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; diC8, 1,2-dioctanoyl-*sn*-glycerol; GST, glutathione transferase; TBS+T, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; PI(3,4,5)P$_3$, phosphatidylinositol 3,4,5-trisphosphate.

ylation 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. $PI(4,5)P_2$, one of the most abundant phosphoinositides in the cell membrane, regulates many cellular events such as cytoskeleton remodeling, vesicle transport, and ion channel or transporter function (Suh and Hille, 2005; Di Paolo and De Camilli, 2006; Gamper and Shapiro, 2007).

In the present study, we explored the interaction of $PI(4,5)P_2$ with native and recombinant $P2X_1$ receptor-channels and its physiological consequences. We report here, using the isolated rat mesenteric artery preparation, a strong modulation of $P2X_1$-mediated vascular reactivity by wortmannin-induced $PI(4,5)P_2$ depletion. Using two-electrode voltage-clamp and inside-out patch recordings in the *Xenopus laevis* oocyte expression system, we also provide functional evidence that $PI(4,5)P_2$ modulates $P2X_1$ current amplitude, recovery, and speed of activation and inactivation and that it is required for the $5\text{-}HT_{2A}$-mediated potentiation of $P2X_1$ currents. Finally, using an in vitro lipid binding assay and mutagenesis, we show that the proximal C-terminal region of $P2X_1$ subunit, containing the critical basic residue Lys_{364}, directly interacts with several anionic phospholipids, including $PI(4,5)P_2$.

Materials and Methods

Reactivity of Isolated Rat Mesenteric Arteries. All procedures were as described previously (Elhusseiny and Hamel, 2000; Tong et al., 2005). In brief, rats were sacrificed by cervical dislocation, and the mesenteric arteries were isolated and collected in cold (4°C) Krebs' solution, pH 7.4, containing 118 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. Arterial segments (approximately 2 mm in length) were transferred to a small superfusion chamber (5 ml; Living Systems Instrumentation, Inc., Burlington, VT), cannulated on a glass micropipette (~80 μm in diameter) at one end, sealed to another glass micropipette at the other end, and filled with oxygenated (5% CO_2 and 95% O_2) Krebs' solution (37°C; pH 7.4). Intraluminal pressure was maintained at 60 mm Hg, and vessels were superfused with Krebs' solution and allowed to stabilize and acquire basal tone. Online measurements of intraluminal diameter were performed using a closed circuit video system coupled with a video caliper. Direct abluminal application of 10 μM α,β-meATP was performed on vessels pretreated or not with the PI3/4-kinases inhibitor wortmannin (100 nM or 1 μM). In the wortmannin experiments, vessels were rapidly washed with fresh Krebs' solution before application of α,β-meATP.

Expression of Recombinant $P2X_1$ Receptors in *Xenopus laevis* Oocytes. Ovary lobes were surgically removed from *X. laevis* frogs deeply anesthetized by immersion in 0.2% Tricaine (Sigma-Aldrich, St. Louis, MO). After treatment with type I collagenase in calcium-free oocyte Ringer's medium for 2 h at room temperature, stage V to VI oocytes were manually defolliculated. Wild-type or mutant rat $P2X_1$ subunit and rat $5\text{-}HT_{2A}$ 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 $P2X_1$ alone, 25 ng of cRNA was injected in each oocyte. In coexpression experiments, 25 ng of $P2X_1$ and 25 ng of $5\text{-}HT_{2A}$ cRNA were injected. Oocytes were kept at 19°C in Barth solution

containing 50 μg/ml gentamicin and 1.8 mM $CaCl_2$ for 48 h before recording.

Electrophysiology. Two-electrode voltage-clamp recordings in *X. laevis* oocytes expressing $P2X_1$ 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 perfused at a flow rate of 10 to 12 ml/min with Ringer's solution, pH 7.4, containing 115 mM NaCl, 5 mM NaOH, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES. Oocytes were held at −60 mV during recording. Unless specified, stimulations with 10 μM ATP (Sigma-Aldrich) dissolved in Ringer's solution were applied consecutively at 5-min intervals, and $P2X_1$ responses were stable after the second application of ATP. When wortmannin was used, oocytes were incubated for 2 h in Barth's solution containing 100 nM or 35 μM wortmannin, or vehicle (0.3% dimethyl sulfoxide), before recording. For experiments involving coexpression of $5\text{-}HT_{2A}$ 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 $P2X_1$ peak current. The phospholipids diC8-$PI(4,5)P_2$ or diC8-$PI(3,4,5)P_3$ (Echelon Biosciences Inc., Salt Lake City, UT) were injected (20 nl; 10 mM) in the cytoplasm of oocytes 30
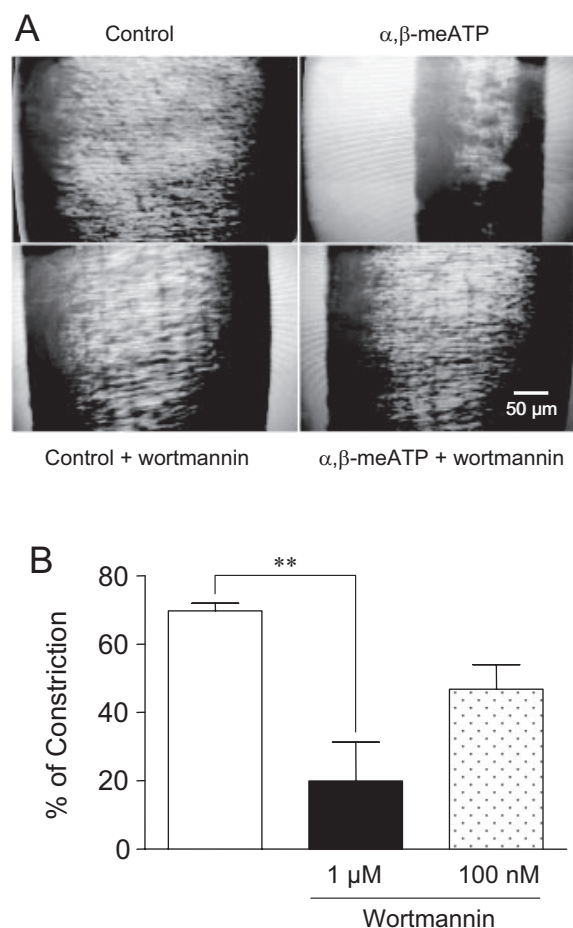
Fig. 1. $P2X_1$ constrictive responses are sensitive to wortmannin. A, representative video recordings of changes in rat mesenteric artery diameter. Control, vessel diameter measured at baseline tone. α,β-meATP, same vessel 1 min after application of 10 μM α,β-meATP. Control + wortmannin, vessel diameter measured at baseline tone 30 min after application of 1 μM wortmannin. α,β-meATP + wortmannin: same vessel, 1 min after application of 10 μM α,β-meATP. B, constriction responses elicited by application of the $P2X_1$ agonist 10 μM α,β-meATP on rat mesenteric artery and their partial blockade after treatment with 1 μM wortmannin ($n = 3–6$; ∗∗, $p < 0.01$).

min before final recording. For all the calculations of final drug concentration, we estimated the oocyte cell volume at 1 μl.

Inside-out macropatch recordings in *X. laevis* oocytes expressing P2X$_1$ channels were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). pClamp 9 was used for data acquisition and analysis. The sampling rate was 2 kHz. A ramp protocol from −100 to +100 mV was applied once per second, and current amplitudes were acquired at −80 mV. Electrodes with resistance of 0.5 to 1.0 MΩ were used. The internal (bath) solution contained 96 mM KCl, 0.5 mM EGTA, and 10 mM HEPES, pH 7.4. The external (pipette) solution contained also 1.8 mM CaCl$_2$, 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 P2X$_1$ (Ile$_{356}$–Ala$_{371}$) flanked by BamHI-EcoRI ends were subcloned into pGEX-2T vector for production of wild-type or mutant GST-P2X$_1$ fusion proteins, before purification on glutathione-Sepharose resin. Lipid binding analysis of GST-P2X$_1$ 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 P2X$_1$ or GST alone were used as negative con-

trols. The membranes were blocked with TBS+T solution supplemented with 3% BSA for 1 h at room temperature, then incubated overnight with 5 μg/ml 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 h at room temperature. Repeated washes with TBS+T were followed by 1-h incubation with the secondary antibody horseradish peroxidase-labeled goat anti-mouse (1:5000) in TBS+T, 3% BSA at room temperature for detection of GST-P2X$_1$ 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)P$_2$ Depletion Decreases P2X$_1$-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 μM) to vessel segments led to strong muscle constriction
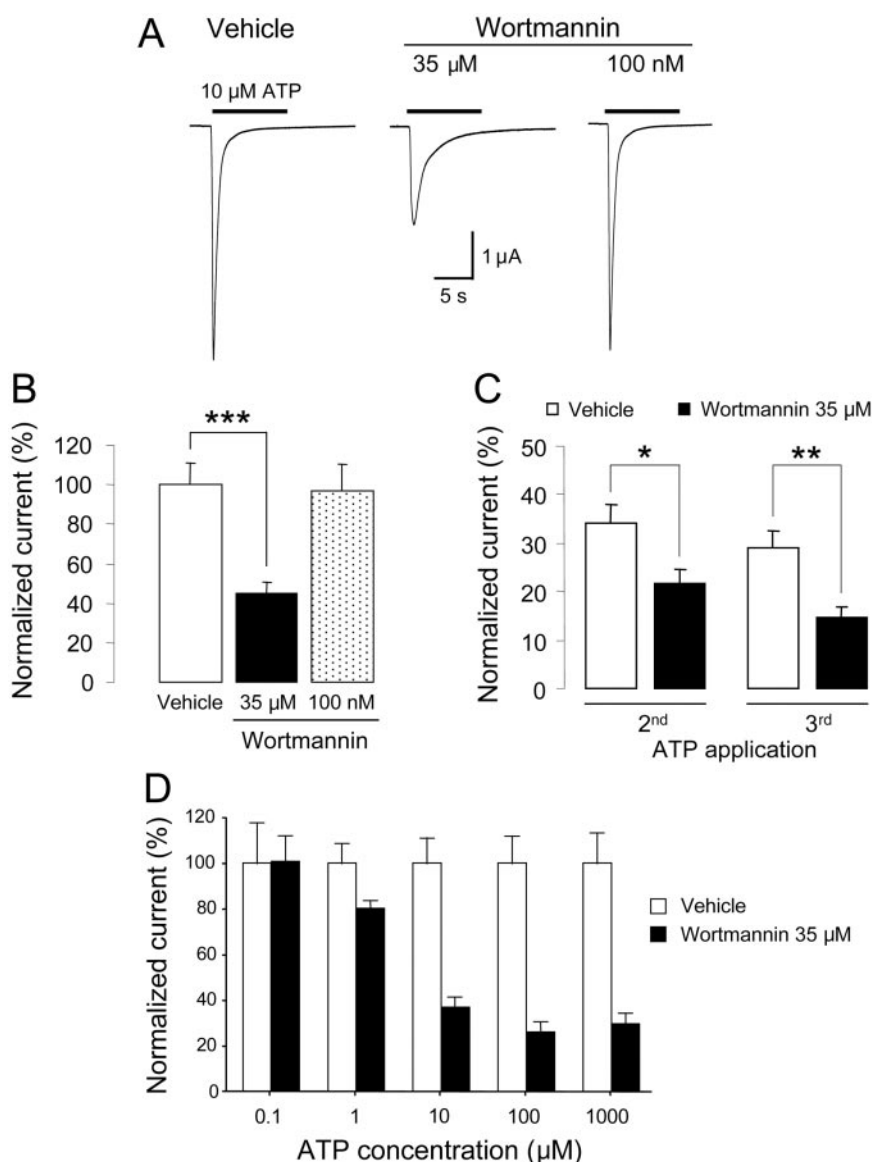

Fig. 2. Inhibitory effect of wortmannin on recombinant P2X$_1$ current responses. A, representative ATP-evoked inward currents in *X. laevis* oocytes expressing P2X$_1$ receptors. High (35 μM) but not low (100 nM) concentration of wortmannin decreases P2X$_1$ peak current. B, quantitative results ($n = 9$–17; ∗∗∗, $p < 0.001$). C, treatment with 35 μM wortmannin decreases recovery of P2X$_1$ currents after 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).

A Vehicle

10 μM ATP   1 μM 5-HT
3rd   4th

1 μA

5 min   0.5 μA
                                          5 s

B 35 μM Wortmannin

10 μM ATP   1 μM 5-HT
3rd   4th

0.2 μA
5 s

C 100 nM Wortmannin

10 μM ATP   1 μM 5-HT
3rd   4th

1 μA

0.5 μA
5 s

D

□ before 5-HT
■ after 5-HT

Normalized current (%)
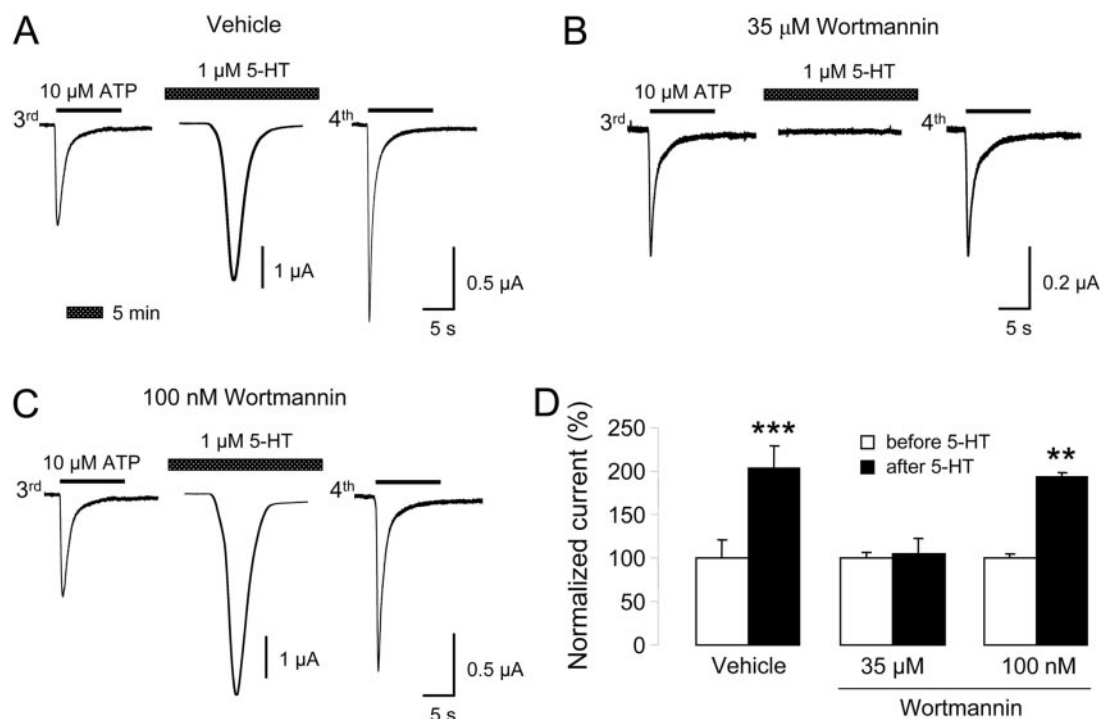
Vehicle   35 μM   100 nM
          Wortmannin

Fig. 3. Wortmannin blocks 5-HT$_{2A}$-mediated recovery of P2X$_1$ currents. A, representative ATP-evoked (third and fourth application) and 5-HT-evoked inward currents from oocytes coexpressing P2X$_1$ and 5-HT$_{2A}$ receptors. A 5-min application of 5-HT accelerates recovery of P2X$_1$ current amplitudes. B, traces show blockade of accelerated recovery of P2X$_1$ current responses by preincubation with 35 μM wortmannin (inhibition of PI3- and PI4-kinases). Note that high wortmannin concentration also suppresses 5-HT-evoked Ca^{2+}-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 P2X$_1$ current responses, normalized to the responses before 5-HT application ($n = 5$–7; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

A

10 μM ATP

Wortmannin
35 μM

Wortmannin 35 μM
+ PI(4,5)P$_2$

1 μA
5 s

B

Peak current (μA)

∗∗∗

Control   +PBS   + PI(4,5)P$_2$ + PI(3,4,5)P$_3$
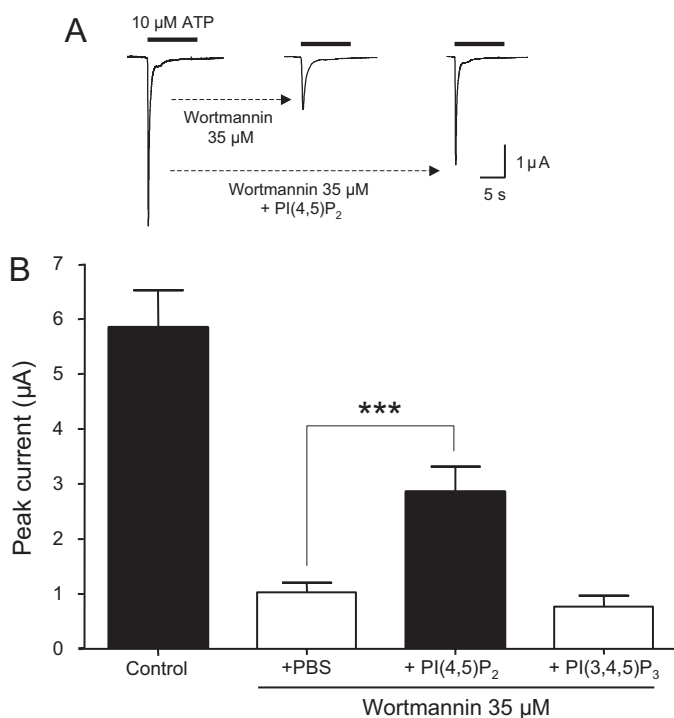   Wortmannin 35 μM

Fig. 4. PI(4,5)P$_2$ specifically rescues P2X$_1$ current amplitude after 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)P$_2$ into oocytes expressing P2X$_1$ receptors. B, quantitative summary of the results, showing the lack of rescue when diC8-PI(3,4,5)P$_3$ is injected ($n = 9$; ∗∗∗, $p < 0.001$).

responses (69 ± 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 P2X$_1$-mediated constrictive responses. When the mesenteric vessels were incubated with 1 μM wortmannin for 1 h, α,β-meATP-mediated constriction was significantly decreased to 28 ± 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 ± 9% of α,β-meATP-mediated constriction, $n = 3$; Fig. 1B).

PI(4,5)P$_2$ Depletion Reduces P2X$_1$ Current Amplitude and Recovery in *X. laevis* Oocytes without Changing Its Sensitivity to ATP. We studied the effects of altering PI(4,5)P$_2$ levels on ATP-evoked currents in *X. laevis* oocytes expressing recombinant P2X$_1$ receptor-channels. The oocytes were pretreated with vehicle or wortmannin at high (35 μM) or low (100 nM) concentration for 2 h before they were stimulated with 10 μM ATP (EC$_{50}$ = 1 μM) for 10 s. We observed that high but not low concentration of wortmannin significantly reduced ATP-induced current amplitudes (45.1 ± 5.5% of control, $n = 23$) as illustrated by the representative traces and quantitative results in Fig. 2, A and B, respectively. Desensitization of P2X$_1$ responses after repeated applications of ATP was significantly stronger in oocytes incubated with 35 μM wortmannin: second response = 21 ± 3% of initial response; third response = 14 ± 2% ($n = 14$), compared with second response = 34 ± 4%; third response = 29 ± 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

μM) was applied at 0.1 μM, the treatment with 35 μM wortmannin had no effect on the current amplitude (3.03 $\pm$ 0.5 μA in vehicle-treated oocytes, $n = 6$, compared with 3.05 $\pm$ 0.3 μA in wortmannin-treated oocytes; $n = 9$). A maximal inhibitory effect was reached at 100 μM ATP (27 $\pm$ 5% of control; $n = 9$). These results indicated that depletion of phosphoinositides with 35 μM 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 G$_q$-coupled 5-HT$_{2A}$ receptors were exposed to Ringer's solution or 1 μM 5-HT for 5 min between the third and fourth ATP applications. Under these conditions, 5-HT transiently elicited inward currents (Fig. 3, A and C), sometimes oscillatory in nature, indicating secondary activation of endogenous Ca^{2+}-dependent chloride currents. Application of 5-HT significantly potentiated P2X₁ current responses, as reported previously (Ase et al., 2005) and illustrated in Fig. 3A (see also Fig. 3D for quantitative results). However, 2 h of preincubation with 35 μM wortmannin blocked the accelerated recovery of P2X₁ currents after 5-HT$_{2A}$ activation (Fig. 3, B and D). Lower concentration of wortmannin (100 nM) did not have any significant effect on 5-HT-induced modulation of P2X₁ currents (Fig. 3, C and 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 200 μM diC8-PI(4,5)P₂ 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 μA ($n = 8$) compared with 1.0 $\pm$ 0.2 μA in PBS-injected oocytes ($n = 14$) (Fig. 4, A and 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, 200 μM diC8-PI(3,4,5)P₃ injections did not have any rescuing effect on the current amplitudes of P2X₁ receptors after 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 Fig. 5A, treatment with high concentration of wortmannin clearly slowed down both the 10 to 90% rise time of the activation phase and the inactivation time (one-exponential fit) of P2X₁ current responses. The 10 to 90% rise time was 0.17 $\pm$ 0.01 s ($n = 9$) in control conditions and 0.25 $\pm$ 0.03 s ($n = 9$) after wortmannin. The time constant (τ) of inactivation was 0.92 $\pm$ 0.07 s ($n = 9$) in control conditions and 1.42 $\pm$ 0.14 s ($n = 9$) after wortmannin. We observed that injection of PI(4,5)P₂ com-
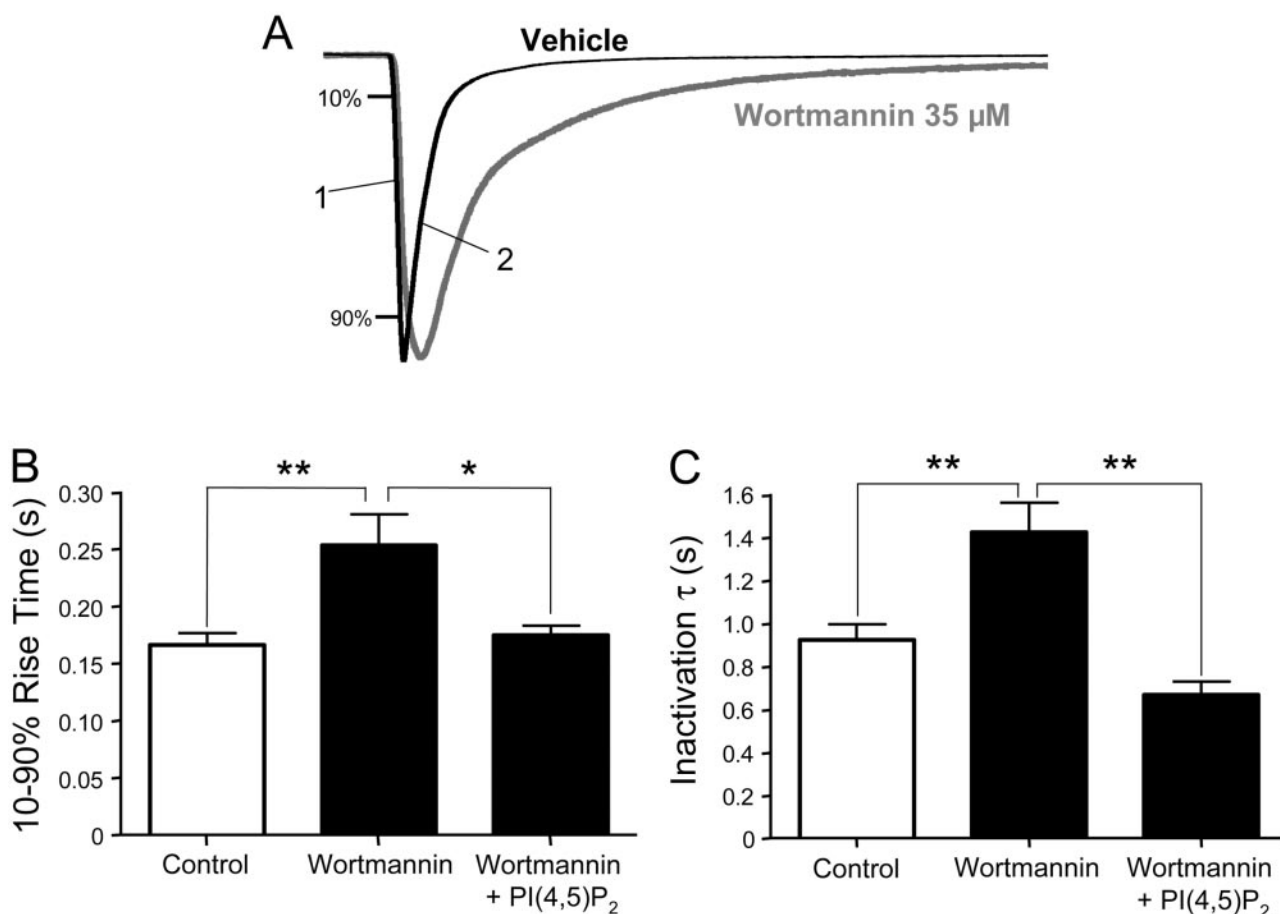

Fig. 5. PI(4,5)P₂ depletion slows down activation and inactivation of P2X₁ currents. A, representative traces showing slower activation (1) and inactivation (2) of ATP-evoked P2X₁ inward current after wortmannin treatment. B, quantitative results show the complete rescue of kinetic parameters of P2X₁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

pletely restored the kinetic parameters of $P2X_1$ responses to their control levels (Fig. 5, B and C).

$PI(4,5)P_2$ Activates $P2X_1$ Current Responses in Excised Macropatches. To test direct effects of phosphoinositides on heterologously expressed $P2X_1$ receptors, we applied diC8-$PI(4,5)P_2$ to inside-out macropatch membranes from *X. laevis* oocytes. Upon patch excision, a fast rundown of the current was observed. Application of 100 μM polylysine, which sequesters $PI(4,5)P_2$, stabilized the $P2X_1$ currents to basal levels (Fig. 6A). Subsequent application of 5 μM diC8-$PI(4,5)P_2$ strongly activated $P2X_1$ currents (Fig. 6A). A quantitative analysis shows significantly larger amplitude ($\sim$2-fold) for $P2X_1$ currents in the presence of diC8-$PI(4,5)P_2$, compared with rundown baseline levels (Fig. 6B).

The Single Lysine Lys_{364} in the C Terminus of $P2X_1$ 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 $P2X_1$ 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 $P2X_2$ receptor (Fujiwara and Kubo, 2006; Zhao et al., 2007). No specific binding was observed when a GST-$P2X_1$ fusion protein expressing the N-terminal peptide Phe_{12}-Val_{29} was tested (Fig. 7B). However the GST-$P2X_1$ fusion protein

containing the C-terminal peptide Ile_{356}-Ala_{371} selectively bound to several phospholipids, including PI(3)P, PI(4)P, PI(5)P, $PI(3,5)P_2$, $PI(4,5)P_2$, $PI(3,4,5)P_3$, phosphatidylserine, and to a lesser extent, $PI(3,4)P_2$ and phosphatidic acid, 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 Lys_{364} is a critical determinant for interaction with phosphoinositides. As shown in Fig. 7C, ATP-evoked current amplitude was smaller in oocytes expressing the mutant receptor $P2X_1$ K364Q (4.94 $\pm$ 0.69 μA; $n = 10$) compared with wild-type receptor (8.27 $\pm$ 0.99 μA; $n = 10$). Moreover, $P2X_1$ K364Q receptors showed higher sensitivity to depletion of $PI(4,5)P_2$ induced by 35 μM wortmannin (13.70 $\pm$ 1.80% of control response; $n = 19$) than wild-type receptors (44.88 $\pm$ 10.13% of control response; $n = 9$), confirming a decrease in $PI(4,5)P_2$ binding affinity (Fig. 7D).

Discussion

The homomeric $P2X_1$ purinoceptor is an ATP-gated receptor-channel mainly expressed in smooth muscles of the genitourinary 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 $P2X_1$ responses by phosphoinositides and its physiological relevance because α,β-meATP-evoked $P2X_1$-mediated constrictions of the rat mesenteric artery are sensitive to the pharmacological depletion of $PI(4,5)P_2$. We were able to reconstitute in the *X. laevis* oocyte expression system the modulation of $P2X_1$-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 $P2X_1$ current responses with high (micromolar) 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 $P2X_1$ receptors to ATP because 1) the inhibition was unsurmountable at supramaximal concentrations of ATP, 2) maximal current amplitude was reached at 10 μM ATP after wortmannin treatment as in control conditions, and 3) wortmannin had no effect on $P2X_1$ currents induced by 0.1 μM ATP. We can conclude that ATP becomes a partial agonist for $P2X_1$ when $PI(4,5)P_2$ levels are depleted. In other words, minimal $PI(4,5)P_2$ levels are required for the expression of a full $P2X_1$ response to ATP. Recovery of $P2X_1$ responses was also found sensitive to treatment with high concentration of wortmannin, suggesting that desensitization of $P2X_1$ receptor-channels is regulated by $PI(4,5)P_2$.

We and others have reported the potentiation of $P2X_1$ responses by activation of G_q-coupled receptors (Vial et al., 2004; Ase et al., 2005), so we tested the impact of wortmannin on the modulation of $P2X_1$ responses by G_q-coupled 5-HT_{2A} receptors. The lack of potentiation of $P2X_1$ responses after blockade of PI4-kinases by 35 μM wortmannin, as well as the absence of effect of PI3-kinases blockade by 100 nM wortmannin, supported a critical role for $PI(4,5)P_2$ as phos-
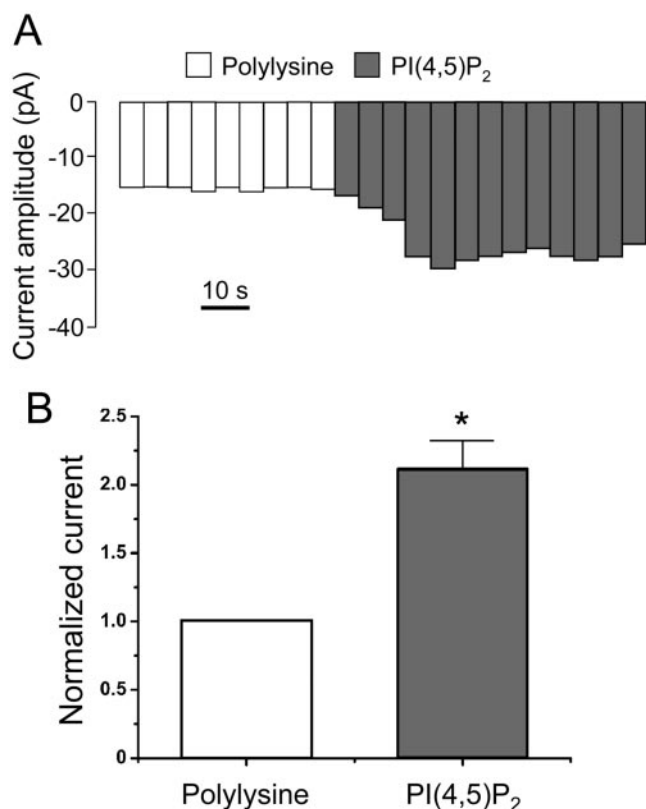

Fig. 6. $PI(4,5)P_2$-activated P2X currents in excised macropatches from *X. laevis* oocytes. A, averaged current amplitudes during 5-s intervals at -80 mV. Currents were evoked by ramps from -100 to $+100$ mV. The internal pipette solution contained 20 μM ATP. Polylysine is applied on the intracellular side at 100 μM. B, quantitative results show rescue of $P2X_1$ currents from ATP-induced rundown by 5 μM diC8-$PI(4,5)P_2$ application ($n = 4$–9; $*$, $p < 0.05$).

pholipase C substrate in the cross-talk between 5-HT$_{2A}$ and P2X$_1$ receptors. The effectiveness of the blockade of PI4-kinases and PI(4,5)P$_2$ synthesis was confirmed by the suppression of inositol 1,4,5-trisphosphate-induced Ca^{2+}-dependent chloride currents, triggered by 5-HT, after treatment with 35 μM wortmannin. Stimulation of the phospholipase C-coupled platelet-derived growth-factor tyrosine kinase receptor has been shown to decrease PI(4,5)P$_2$-sensitive P2X$_7$ currents (Zhao et al., 2007); however, we did not observe any decrease of P2X$_1$ responses after 5-HT$_{2A}$ stimulation. This suggests that a transient stimulation of G$_q$-coupled receptors does not significantly deplete the steady-state levels of PI(4,5)P$_2$ 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)P$_2$ in modulating P2X$_1$ channels responses is directly supported by the fact that addition of PI(4,5)P$_2$, and not PI(3,4,5)P$_3$, significantly rescued the amplitude of P2X$_1$ currents after blockade of PI4-kinases with 35 μM wortmannin. Decreased levels of phosphoinositides not only affected the amplitude of P2X$_1$ currents but also their kinetics, because wortmannin clearly slowed down their 10 to 90% rise time and increased their time constant of inactivation. Addition of intracellular PI(4,5)P$_2$ after treatment with wortmannin normalized the kinetics of the P2X$_1$ currents back to their control values. Under basal conditions, however, increasing PI(4,5)P$_2$ levels by direct injection into the cytoplasm affected neither basal P2X$_1$ current amplitudes nor activation/inactivation kinetics. Thus, at least in oocytes, endogenous

PI(4,5)P$_2$ levels saturate the P2X$_1$ receptor-channels expressed at the cell surface.

Our results using inside-out macropatches excised from P2X$_1$-expressing oocytes provided evidence for the sensitivity of P2X$_1$ receptor-channels to direct application of PI(4,5)P$_2$. P2X$_1$ shows little activity upon patch excision because of a rapid rundown resulting from receptor desensitization and dephosphorylation of PI(4,5)P$_2$ 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)P$_2$ (Lopes et al., 2002; Suh and Hille, 2002, 2005; Zhang et al., 2003). Application of exogenous PI(4,5)P$_2$ on the intracellular side of the patch rescued P2X$_1$ currents from rundown, indicating the requirement of PI(4,5)P$_2$ for full P2X$_1$ function and arguing against trafficking to the surface as the mechanism for PI(4,5)P$_2$-induced potentiation of P2X$_1$ currents.

Searching for phospholipid-binding motifs in the intracellular sequences of P2X$_1$ subunits, we confirmed the direct binding of PI(4,5)P$_2$ to the C-terminal domain Ile$_{356}$-Ala$_{371}$. 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 (five lysines and one arginine), and we have found that the single lysine at position 364 plays a critical role in these interactions. The mutant receptor P2X$_1$ K364Q re-
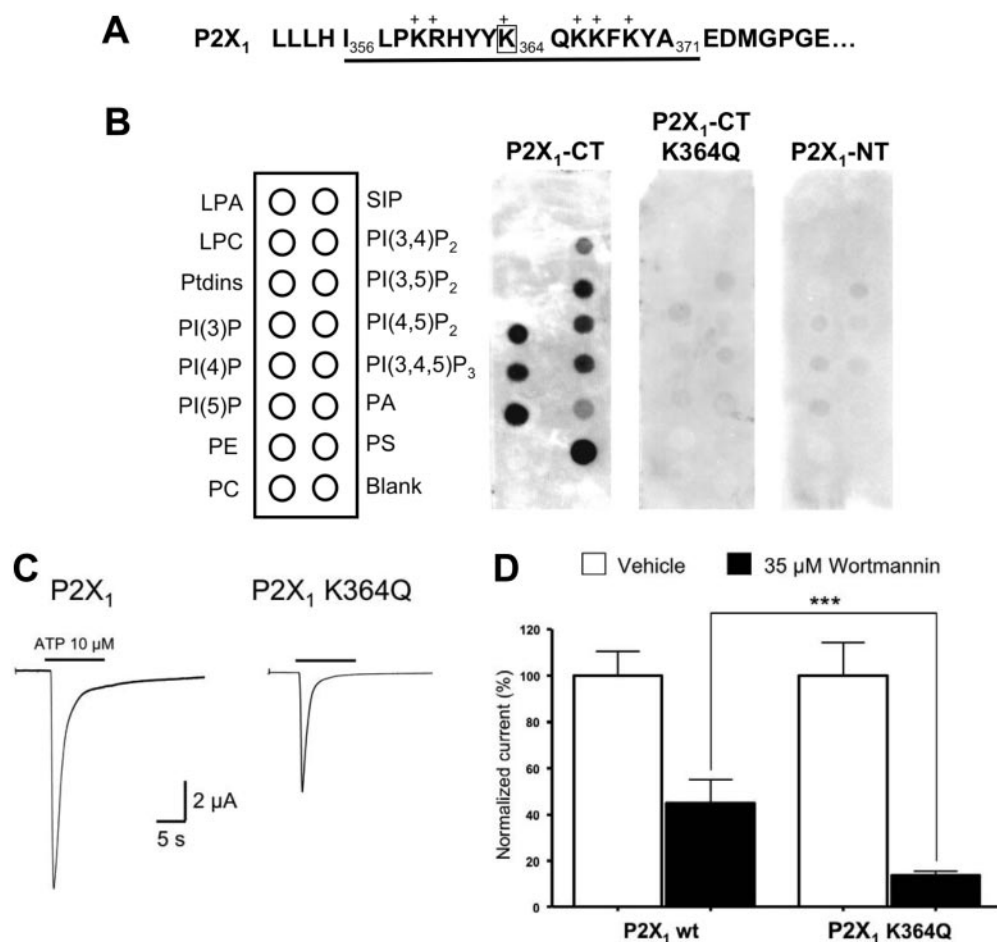
Fig. 7. Lysine Lys$_{364}$ in the proximal C-terminal domain of P2X$_1$ subunit is a critical determinant for phosphoinositide binding and sensitivity to wortmannin. A, sequence of the proximal C-terminal domain of rat P2X$_1$ subunit. B, the GST construct containing the proximal C-terminal domain Ile$_{356}$-Ala$_{371}$ of the P2X$_1$ subunit binds to several anionic phosphoinositides, including PI(4,5)P$_2$. Binding is suppressed when the single lysine Lys$_{364}$ is replaced with glutamine. No binding is detected when using the GST construct containing the N-terminal domain of P2X$_1$ (P2X$_1$-NT) as negative control. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. C, representative traces showing that the mutant receptor P2X$_1$ K364Q carries smaller ATP-evoked currents than the wild-type P2X$_1$ receptor (n = 10; *, $p < 0.05$). D, the mutant receptor P2X$_1$ K364Q is also more sensitive than the wild-type receptor to depletion of PI(4,5)P$_2$ induced by 35 μM wortmannin (n = 9–19; ***, $p < 0.001$).

mains functional but with a lower binding affinity for $PI(4,5)P_2$, indicated by a higher sensitivity to wortmannin-induced depletion, analogously to mutants of other $PI(4,5)P_2$-sensitive channels (Roháes et al., 2005). The fact that the K364Q mutation in the functional $P2X_1$ receptor-channel did not lead to complete loss of $PI(4,5)P_2$ binding and insensitivity to wortmannin suggests that other determinants contribute to phosphoinositides binding. It is noteworthy that the C-terminal sequence of $P2X_1$ also showed strong affinity for other anionic phospholipids. $PI(4,5)P_2$ 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 (Zhainazarov 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 $P2X_2$ (Fujiwara and Kubo, 2006). Therefore, this short domain seems to be of general importance for the regulation of P2X function by phosphoinositides.

In conclusion, we have provided strong evidence that the phospholipid $PI(4,5)P_2$ is a physiological ligand of $P2X_1$ receptor-channels, and our results suggest a novel mode of regulation of ionotropic $P2X_1$ responses by the various metabotropic pathways linked to phosphoinositide metabolism.

References

Ase AR, Raouf R, Bélanger D, Hamel E, and Séguéla P (2005) Potentiation of P2X1 ATP-gated currents by 5-hydroxytryptamine 2A receptors involves diacylglycerol-dependent kinases and intracellular calcium. *J Pharmacol Exp Ther* **315:**144–154.

Banks FC, Knight GE, Calvert RC, Thompson CS, Morgan RJ, and Burnstock G (2006) The purinergic component of human vas deferens contraction. *Fertil Steril* **85:**932–939.

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87:**659–797.

Di Paolo G and De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443:**651–657.

Elhusseiny A and Hamel E (2000) Muscarinic—but not nicotinic—acetylcholine receptors mediate a nitric oxide-dependent dilation in brain cortical arterioles: a possible role for the M5 receptor subtype. *J Cereb Blood Flow Metab* **20:**298–305.

Fujiwara Y and Kubo Y (2006) Regulation of the desensitization and ion selectivity of ATP-gated P2X2 channels by phosphoinositides. *J Physiol* **576:**135–149.

Gamper N and Shapiro MS (2007) Regulation of ion transport proteins by membrane phosphoinositides. *Nat Rev Neurosci* **8:**921–934.

Hansen MA, Balcar VJ, Barden JA, and Bennett MR (1998) The distribution of single P2x1-receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder. *J Neurocytol* **27:**529–539.

Hilgemann DW (2007) Local PIP(2) signals: when, where, and how? *Pflugers Arch* **455:**55–67.

Huang CL, Feng S, and Hilgemann DW (1998) Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gβγ. *Nature* **391:**803–806.

Lopes CM, Zhang H, Rohacs T, Jin T, Yang J, and Logothetis DE (2002) Alterations in conserved Kir channel-PIP2 interactions underlie channelopathies. *Neuron* **34:**933–944.

Mulryan K, Gitterman DP, Lewis CJ, Vial C, Leckie BJ, Cobb AL, Brown JE, Conley EC, Buell G, Pritchard CA, et al. (2000) Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. *Nature* **403:**86–89.

Nakanishi S, Catt KJ, and Balla T (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc Natl Acad Sci U S A* **92:**5317–5321.

North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82:**1013–1067.

Pochynyuk O, Staruschenko A, Tong Q, Medina J, and Stockand JD (2005) Identification of a functional phosphatidylinositol 3,4,5-trisphosphate binding site in the epithelial Na+ channel. *J Biol Chem* **280:**37565–37571.

Roháes T, Lopes CM, Michailidis I, and Logothetis DE (2005) PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci* **8:**626–634.

Smith NC and Burnstock G (2004) Mechanisms underlying postjunctional synergism between responses of the vas deferens to noradrenaline and ATP. *Eur J Pharmacol* **498:**241–248.

Sneddon P (2000) Electrophysiology of autonomic neuromuscular transmission involving ATP. *J Auton Nerv Syst* **81:**218–224.

Srivastava S, Li Z, Lin L, Liu G, Ko K, Coetzee WA, and Skolnik EY (2005) The phosphatidylinositol 3-phosphate phosphatase myotubularin-related protein 6 (MTMR6) is a negative regulator of the Ca2+-activated K+ channel KCa3.1. *Mol Cell Biol* **25:**3630–3638.

Suh BC and Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidyl 4,5-bisphosphate synthesis. *Neuron* **35:**507–520.

Suh BC and Hille B (2005) Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr Opin Neurobiol* **15:**370–378.

Tong XK, Nicolakakis N, Kocharyan A, and Hamel E (2005) Vascular remodeling versus amyloid beta-induced oxidative stress in the cerebrovascular dysfunctions associated with Alzheimer's disease. *J Neurosci* **25:**11165–11174.

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, and Buell G (1994) A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* **371:**516–519.

Vial C, Tobin AB, and Evans RJ (2004) G-protein-coupled receptor regulation of P2X(1) receptors does not involve direct channel phosphorylation. *Biochem J* **382:**101–110.

Vial C and Evans RJ (2005) Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. *J Biol Chem* **280:**30705–30711.

Zhainazarov AB, Spehr M, Wetzel CH, Hatt H, and Ache BW (2004) Modulation of the olfactory CNG channel by Ptdlns(3,4,5)P3. *J Membr Biol* **201:**51–57.

Zhang H, Craciun LC, Mirshahi T, Roháes T, Lopes CM, Jin T, and Logothetis DE (2003) PIP2 activated KCNQ channels and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* **37:**963–975.

Zhang H, He C, Yan X, Mirshahi T, and Logothetis DE (1999) Activation of inwardly rectifying K+ channels by distinct PtdIns(4,5)P2 interactions. *Nat Cell Biol* **1:**183–188.

Zhao Q, Yang M, Ting AT, and Logothetis DE (2007) PIP2 regulates the ionic current of P2X receptors and P2X7 receptor-mediated cell death. *Channels* **1:**46–55.

Address correspondence to: Dr. Philippe Séguéla, Montreal Neurological Institute, 3801 University, Suite 778, Montreal, QC, Canada H3A 2B4. E-mail: philippe.seguela@mcgill.ca