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DIRECT MODULATION OF $P2X_1$ RECEPTOR-CHANNELS BY THE LIPID PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE

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Running Title: Modulation of $P2X_1$ by $PI(4,5)P_2$

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α,β-meATP : α,β-methylene ATP

PI: phosphatidylinositol

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Abstract

The $P2X_1$ 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 $P2X_1$ 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 $PI(4,5)P_2$ with the PI4-kinase inhibitor wortmannin. Using the *Xenopus* oocyte expression system, we provided electrophysiological evidence that lowering $PI(4,5)P_2$ levels with wortmannin significantly decreases $P2X_1$ currents amplitude and recovery. Previously reported modulation of recovery of desensitized $P2X_1$ currents by phospholipase C-coupled $5\text{-}HT_{2A}$ metabotropic receptors was also found 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 $PI(3,4,5)P_3$. 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 K364 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.

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P2X receptors are nonselective cation channels gated by extracellular ATP. P2X subunits ($P2X_{1-7}$) assemble to form either homo- or hetero-trimeric channels, giving rise to a wide range of current phenotypes (North, 2002). Among P2X receptors, the $P2X_1$ 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 (Roberts et al., 2006). For instance, 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, $P2X_1$ receptors currents have been shown to be potentiated by stimulation of co-expressed Gq-coupled $mGluR_1$, $P2Y_{1/2}$ 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 HEK293 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 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. $PI(4,5)P_2$, one of the most abundant phosphoinositide in the cell membrane, regulates many cellular events such as

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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 $PI(4,5)P_2$ 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* oocyte expression system, we also provide functional evidence that $PI(4,5)P_2$ modulates $P2X_1$ current amplitude, recovery, speed of activation and inactivation, and 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 K364, directly interacts with several anionic phospholipids including $PI(4,5)P_2$.

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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 $CaCl_2$, 1 $MgSO_4$, 1 KH_2PO_4, 25 $NaHCO_3$, 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 µm 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 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 µM) was performed on vessels pre-treated 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* 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 $P2X_1$ 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 $P2X_1$ alone 25 ng of

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cRNA were injected in each oocyte. In co-expression 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 of gentamycin and 1.8 mM $CaCl_2$ for 48 h before recording.

Electrophysiology. Two-electrode voltage clamp recordings in *Xenopus* 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 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 $CaCl_2$, and 10 mM HEPES. Oocytes were held at -60 mV during recording. Unless specified, stimulations with 10 μM ATP (Sigma-Aldrich) dissolved in Ringer 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 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 $5\text{-}HT_{2A}$ 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) 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 $P2X_1$ 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

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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 $CaCl_2$, 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 $P2X_1$ (I_{356}–A_{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 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-$P2X_1$ 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.

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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 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 hour, 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 *Xenopus* oocytes without changing its sensitivity to ATP. We studied the effects of altering $PI(4,5)P_2$ levels on ATP-evoked currents in *Xenopus* 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 hours before they were stimulated with 10 μM ATP (EC_{50}= 1 μM) for 10 seconds. 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 Figure 2A and 2B respectively. Desensitization of $P2X_1$ responses following repeated applications of ATP was significantly stronger in oocytes incubated with 35 μM wortmannin: 2^{nd} response= 21 ± 3%, of initial response, 3^{rd} response= 14 ± 2% (n= 14) compared with 2^{nd} response= 34 ± 4%, 3^{rd} response= 29 ± 3% (n= 18) for vehicle-treated

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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 ± 0.5 μA in vehicle-treated oocytes, n= 6, compared to 3.05 ± 0.3 μA in wortmannin-treated oocytes, n= 9). A maximal inhibitory effect was reached at 100 μM ATP (27 ± 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 $P2X_1$ responses via 5-HT_{2A} activation is blocked by wortmannin-induced $PI(4,5)P_2$ depletion. Oocytes coexpressing $P2X_1$ and Gq-coupled 5-HT_{2A} receptors were exposed to Ringer 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. 3A,C), sometimes oscillatory in nature, indicating secondary activation of endogenous Ca^{2+}-dependent chloride currents. Application of 5-HT significantly potentiated $P2X_1$ 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 μM wortmannin blocked the accelerated recovery of $P2X_1$ 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 $P2X_1$ currents (Fig. 3C,D).

Rescue of $P2X_1$ current responses and kinetics by $PI(4,5)P_2$. To study the effect of $PI(4,5)P_2$ on $P2X_1$ receptor-channels expressed in oocytes, we injected diC8-$PI(4,5)P_2$ (200 μM) in the cytoplasm 30 min before recording. Under basal conditions, ATP-evoked $P2X_1$ current amplitudes and kinetics were not affected by the addition of diC8-$PI(4,5)P_2$

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(data not shown). However, in oocytes preincubated with 35 μM wortmannin, diC8-PI(4,5)P_2 addition led to a significant rescue of $P2X_1$ current amplitudes: 2.8 ± 0.5 μA (n= 8) compared to 1.0 ± 0.2 μA in PBS-injected oocytes (n= 14) (Fig. 4A,B). Thus, whereas addition of diC8-PI(4,5)P_2 did not seem to increase ATP-evoked $P2X_1$ currents under basal conditions, it did so when endogenous levels of PI(4,5)P_2 were previously depleted by wortmannin treatment. In contrast, diC8-PI(3,4,5)P_3 (200 μM) injections did not have any rescuing effect on the current amplitudes of $P2X_1$ receptors following wortmannin treatment (Fig. 4B).

In addition, we found that PI(4,5)P_2 levels have a significant impact on the kinetics of $P2X_1$ 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 $P2X_1$ current responses. The 10-90% rise time was 0.17 ± 0.01 s (n= 9) in control conditions and 0.25 ± 0.03 s (n= 9) after wortmannin. The time constant of inactivation was 0.92 ± 0.07 s (n= 9) in control conditions and 1.42 ± 0.14 s (n= 9) after wortmannin. We observed that injection of PI(4,5)P_2 completely restored the kinetic parameters of $P2X_1$ responses to their control levels (Fig. 5B,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 *Xenopus* 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 (~ 2-fold) for $P2X_1$

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currents in the presence of diC8-PI(4,5)P_2, compared to rundown baseline levels (Fig. 6B).

The single lysine K364 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 F_{12}-V_{29} was tested (Fig. 7B). However the GST-P2X_1 fusion protein containing the C-terminal peptide I_{356}–A_{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, PS, and to a lesser extent, PI(3,4)P_2 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 P2X_1 K364Q (4.94 ± 0.69 µA, n= 10) compared to wild-type receptor (8.27 ± 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 ± 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)P_2 binding affinity (Fig. 7D).

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Discussion

The homomeric $P2X_1$ 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 $P2X_1$ responses by phosphoinositides and its physiological relevance since 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 *Xenopus* 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 (μM) 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

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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 Gq-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 Gq-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 PLC substrate in the crosstalk 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 IP_3-induced Ca^{2+}-dependent chloride currents, triggered by 5-HT, after treatment with 35 µM wortmannin. Stimulation of the PLC-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 Gq-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, as wortmannin clearly slowed down their 10-90% rise time and increased their time constant of inactivation. Addition of intracellular $PI(4,5)P_2$ after

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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 did not affect 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 due to a rapid rundown resulting from receptor desensitization and dephosphorylation of $PI(4,5)P_2$ by lipid phophatases (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 carboxy-terminal domain I_{356}-A_{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 (5 lysines and 1 arginine) and we have found that the single lysine K364 plays a critical role in these interactions. The mutant receptor $P2X_1$ K364Q remains functional but with a

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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 (Rohàcs 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. Interestingly, 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 appears to be of general importance for the regulation of P2X function by phosphoinositides.

In conclusion we have provided strong evidence that the phospholipid $PI(4,5)P_2$ 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 phosphoinositides metabolism.

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Footnotes

LPB and ARA contributed equally to this work.

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Figure Legends

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

Figure 2. Inhibitory effect of wortmannin on recombinant $P2X_1$ current responses. (A) Representative ATP-evoked inward currents in *Xenopus* 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 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 $5\text{-}HT_{2A}$-mediated recovery of $P2X_1$ currents. (A) Representative ATP-evoked (3^{rd} and 4^{th} application) and 5-HT-evoked inward currents from oocytes coexpressing $P2X_1$ and $5\text{-}HT_{2A}$ 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)

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Quantification of recovery of $P2X_1$ current responses, normalized to the responses before 5-HT application (n= 5-7, ** $p < 0.01$; *** $p < 0.001$).

Figure 4. $PI(4,5)P_2$ specifically rescues $P2X_1$ 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-$PI(4,5)P_2$ 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, *** $p < 0.001$).

Figure 5. $PI(4,5)P_2$ depletion slows down activation and inactivation of $P2X_1$ currents. (A) Representative traces showing slower activation (1) and inactivation (2) of ATP-evoked $P2X_1$ inward current after wortmannin treatment. (B) Quantitative results show the complete rescue of kinetic parameters of $P2X_1$ currents to normal levels by $PI(4,5)P_2$ delivery into oocytes (n= 9, * $p < 0.05$; ** $p < 0.01$).

Figure 6. $PI(4,5)P_2$-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 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, * $p < 0.05$).

Figure 7. Lysine K364 in the proximal C-terminal domain of $P2X_1$ subunit is a critical determinant for phosphoinositides 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 I_{356}-A_{371} of the $P2X_1$ subunit binds to several anionic phosphoinositides, including $PI(4,5)P_2$. Binding is suppressed when the

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single lysine K364 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, *** $p < 0.001$).

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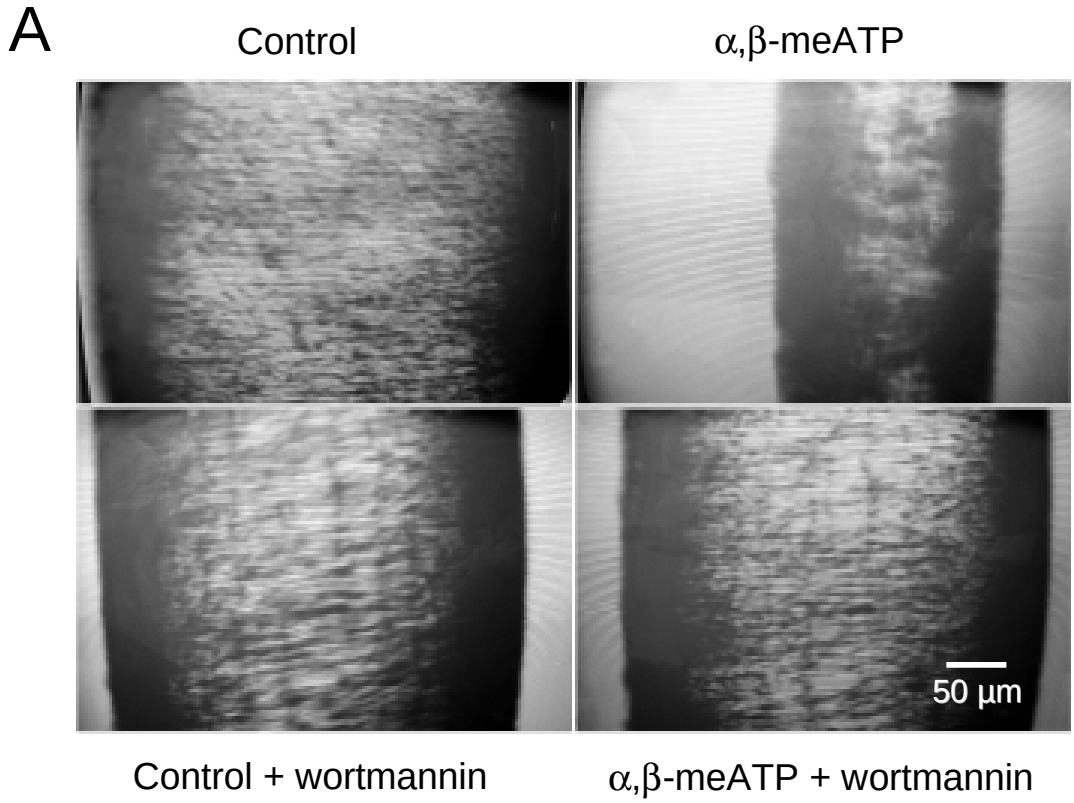
Fig. 1



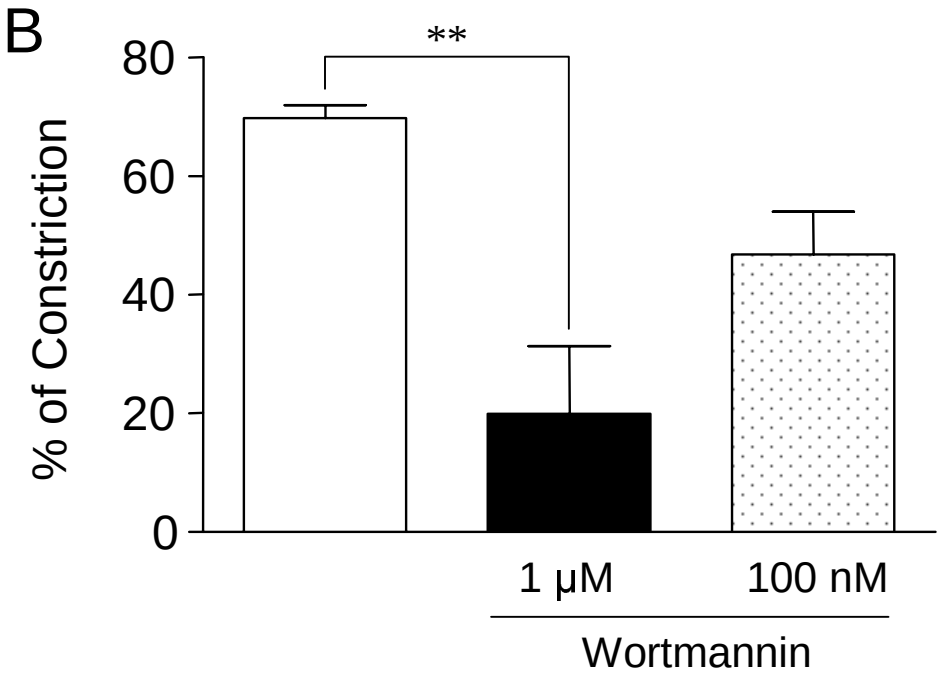


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Fig. 2

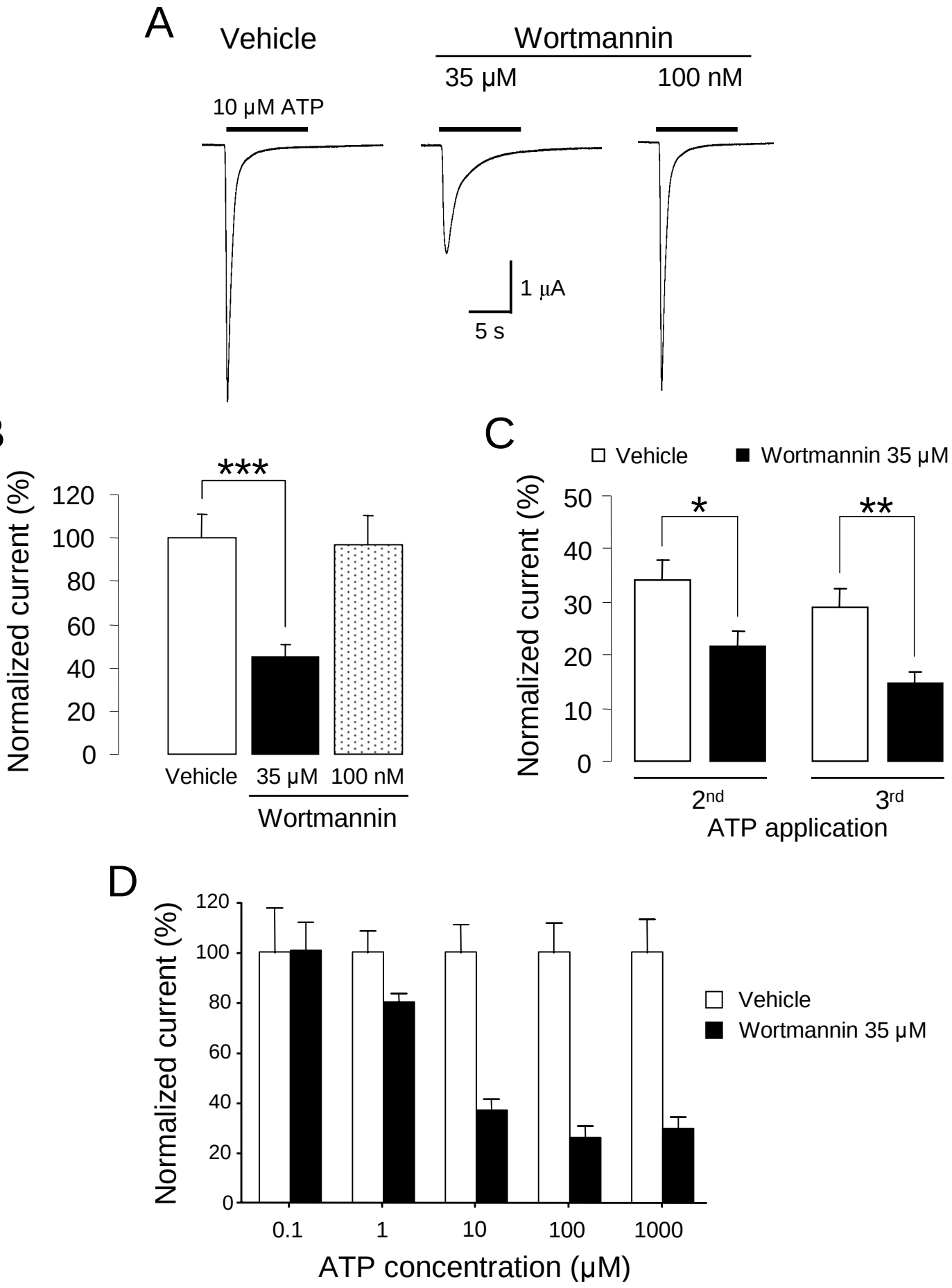

Fig. 3

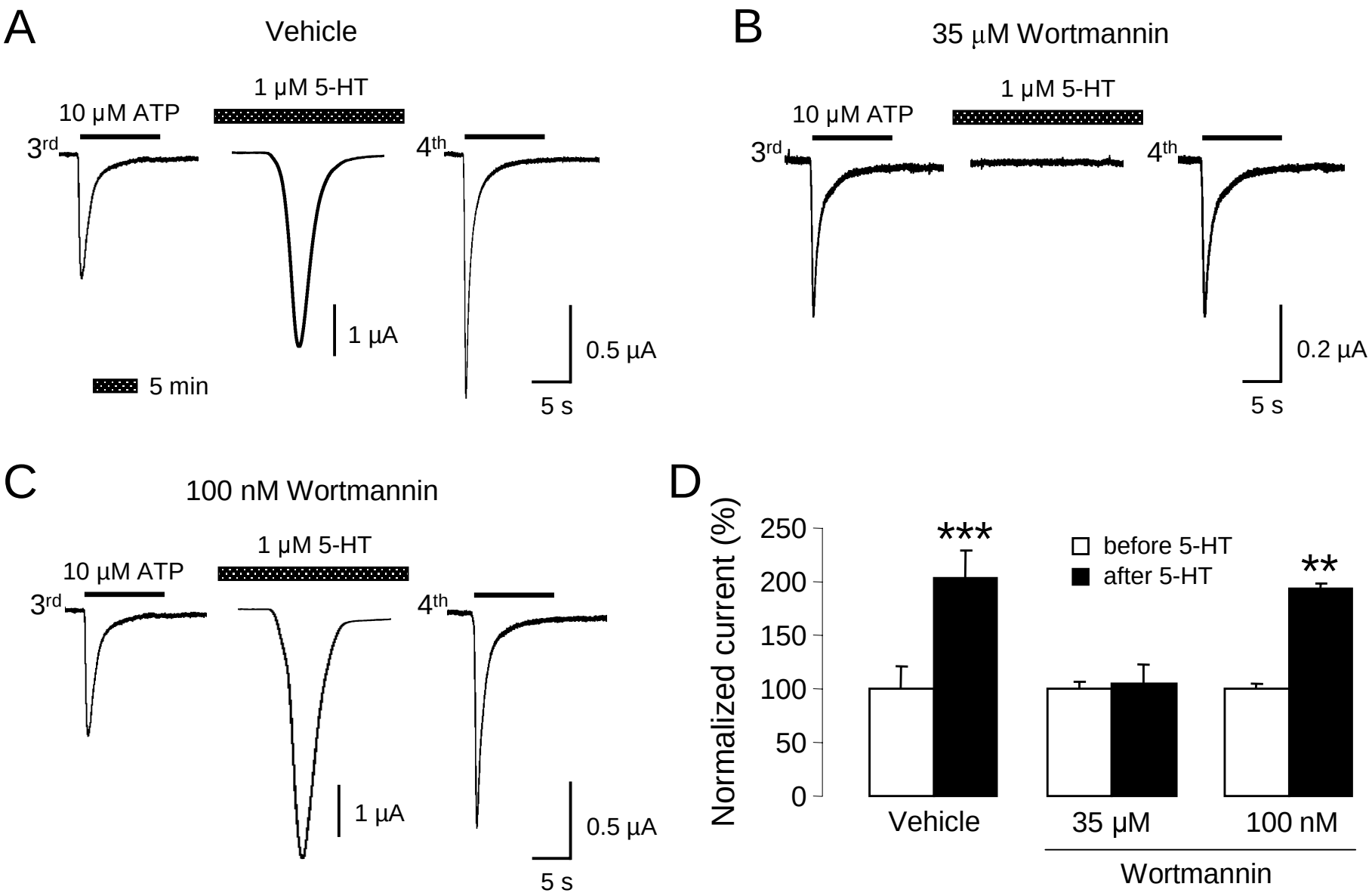

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Fig. 4

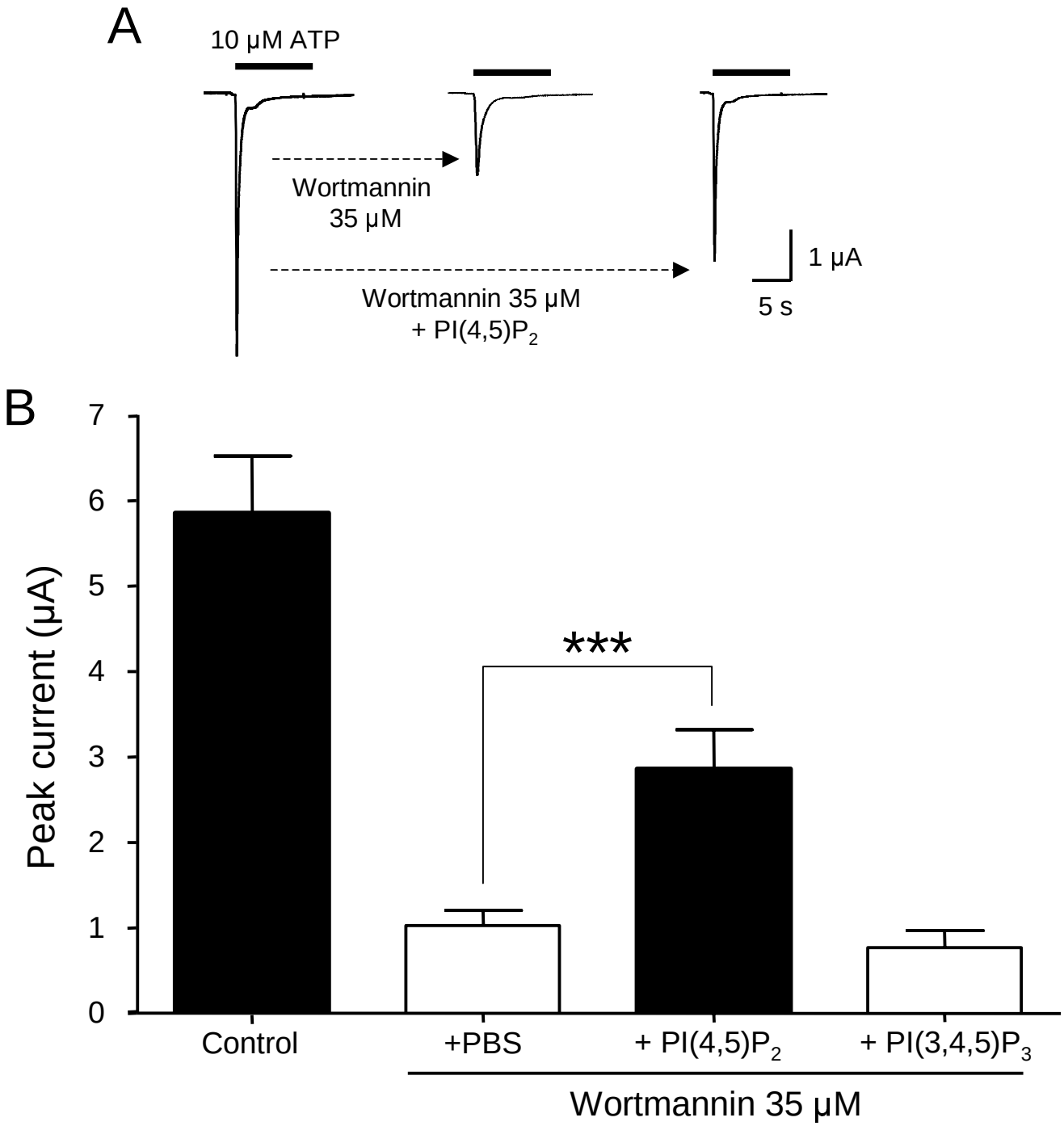

Fig. 5

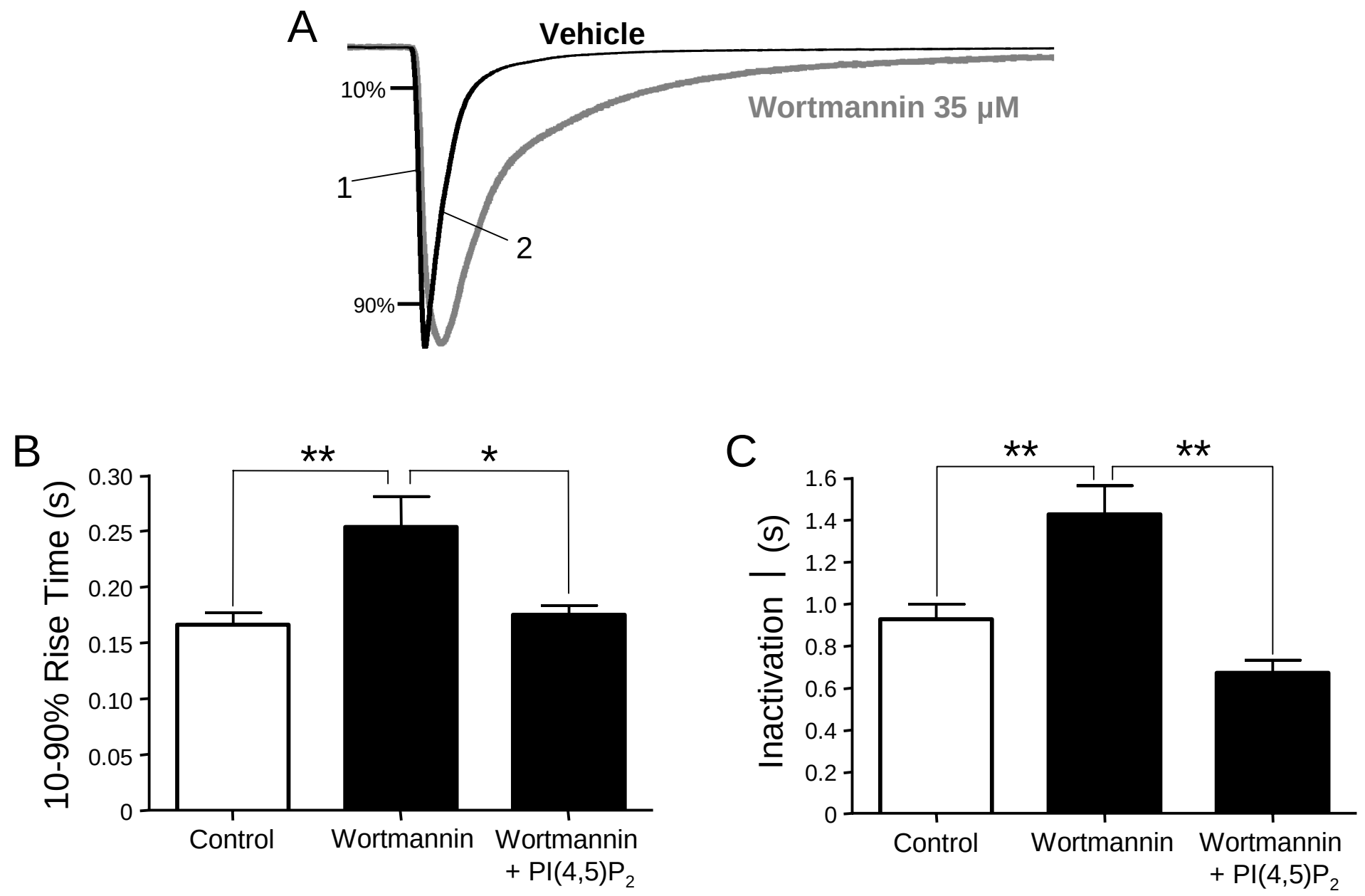

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Fig. 6

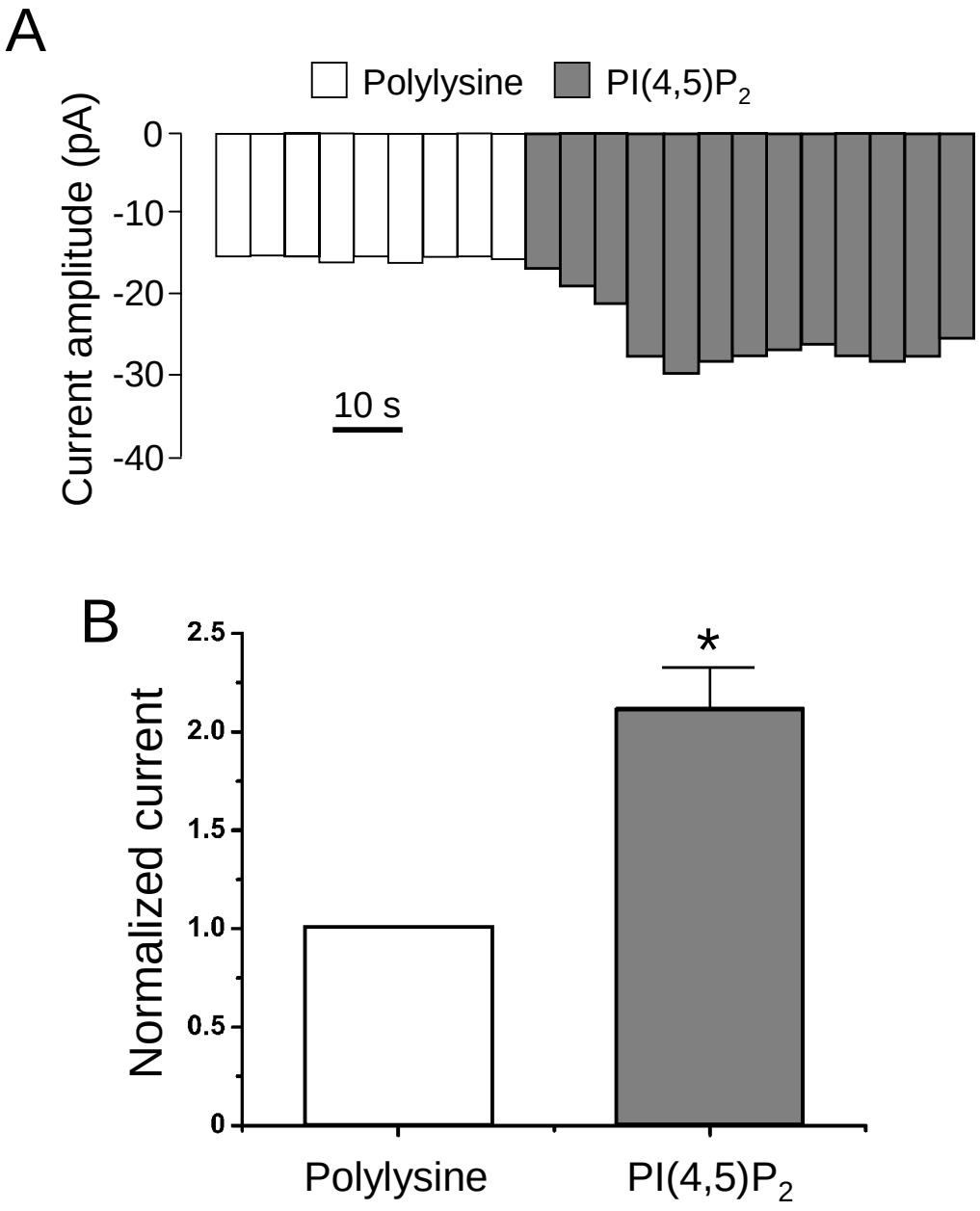

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Fig. 7