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WNK1 Promotes PIP_2 Synthesis to Coordinate Growth Factor and GPCR-G_q Signaling

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Summary

Background—PLC-β signaling is generally thought to be mediated by allosteric activation by G proteins and Ca^{2+}. While availability of the PIP_2 substrate is limiting in some cases, its production has not been shown to be independently regulated as a signaling mechanism. WNK1 protein kinase is known to regulate ion homeostasis and cause hypertension when expression is increased by gene mutations. However, its signaling functions remain largely elusive.

Results—Using diacylglycerol-stimulated TRPC6 and inositol trisphosphate-mediated Ca^{2+} transients as cellular biosensors, we show that WNK1 stimulates PLC-β signaling in cells by promoting the synthesis of PIP_2 *via* stimulation of phosphatidylinositol 4-kinase IIIα. WNK1 kinase activity is not required. Stimulation of PLC-β by WNK1 and by $G\alpha_q$ are synergistic; WNK1 activity is essential for regulation of PLC-β signaling by G_q-coupled receptors and basal input from G_q is necessary for WNK1 signaling via PLC-β. WNK1 further amplifies PLC-β signaling when it is phosphorylated by Akt kinase in response to insulin-like growth factor.

Conclusions—WNK1 is a novel regulator of PLC-β that acts by controlling substrate availability. WNK1 thereby coordinates signaling between G protein and Akt kinase pathways. Because PIP_2 is itself a signaling molecule, regulation of PIP_2 synthesis by WNK1 also allows the cell to initiate PLC signaling while independently controlling the effects of PIP_2 on other targets. These findings describe a new signaling pathway for Akt-activating growth factors, a mechanism for G protein-growth factor crosstalk and a means to independently control PLC signaling and PIP_2 availability.

Introduction

Mammalian WNK kinases are a family of four serine-threonine protein kinases with an atypical kinase active site [1–3]. Mutations in WNK1 and WNK4 cause the autosomal-dominant disease pseudohypoaldosteronism type 2 (PHA2), which is characterized by

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hypertension and hyperkalemia [2]. WNKs regulate various ion transporters through both kinase activity-dependent and -independent mechanisms [3, 4]. For example, WNK1 and 4 phosphorylate and activate Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress-responsive kinase-1 (OSR1), which in turn activate cation-chloride co-transporters NCC and NKCC [5–7]. Through kinase-independent events, WNK1 binds and somehow activates SGK kinase, causing it to activate the epithelial Na^+ channel ENaC [8]; and WNK1 and 4 interact with the endocytic scaffold protein intersectin to enhance endocytosis of the renal K^+ channel ROMK [9]. Dysregulation of ion transport in the kidney contributes to the hypertension and hyperkalemia of PHA2.

The functions of WNKs extend beyond regulation of ion homeostasis. WNK1 is ubiquitously expressed, and homozygous gene inactivation in mice causes cardiovascular developmental arrest and embryonic death [10]. WNK1 activates ERK5, and knockdown of WNK1 decreases activation of ERK5 by epidermal growth factor receptors [11]. WNK2 binds the small GTPases RhoA and Rac1. WNK2 knockdown decreases RhoA activation, but promotes Rac1 activation [12]. The G_q-coupled angiotensin II receptor regulates the activity of the NCC co-transporter through the WNK4 signaling pathway [13], indicating functional interactions between WNK and G protein signaling pathways.

Upstream regulation of the WNKs is incompletely understood, and it is likely that several distinct mechanisms of WNK activation are used in different cells [3, 4]. WNK1 is a substrate of Akt kinase [14, 15], which is a target of phosphatidylinositol 3-kinase (PI3K)-activating growth factors such as insulin and insulin-like growth factors (IGFs). Insulin and IGFs activate PI3K, and its PIP_3 product recruits and activates Akt to phosphorylate WNK1 at threonine-58. It has been reported that Akt-catalyzed phosphorylation of WNK1 at T58 alters the ability of WNK1 to regulate membrane trafficking of ENaC and ROMK channels [8, 16].

Phospholipase C-βs (PLC-βs) are important effectors of G protein-coupled receptor (GPCR)-mediated signaling pathways [17, 18]. They hydrolyze PIP_2, itself a signaling molecule, to form two other second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). PLC-βs are stimulated by Gα subunits of the G_q family, by Gβγ subunits of the G_i's and, in the case of PLC-β2, by Rac [17–19]. The concentration of PIP_2 in the plasma membrane is limiting for PLC-β activity, and re-synthesis of the substrate initiated by phosphatidylinositol-4-kinase (PI4K) after depletion is important for sustained PLC-β signaling [20, 21]. This dependence on substrate concentration agrees with *in vitro* studies showing that PLC activity increases linearly with the concentration of PIP_2 regardless of stimulation by G proteins or Ca^{2+} [22, 23]. However, it is not known whether PIP_2 production can be independently regulated as a signaling mechanism and, if so, by what physiological stimulus.

We show here that WNK1 stimulates PLC-β signaling by promoting the synthesis of PIP_2 *via* stimulation of PI4KIIIα. Stimulation of PLC-β by WNK1 and by $Gα_q$-GTP is thus synergistic, and WNK1 activity is essential for regulation of PLC-β signaling by G_q-coupled receptors. Phosphorylation of WNK1 by the PI3K-Akt kinase cascade enhances the ability of WNK1 to synergistically regulate PLC-β signaling *via* synthesis of PIP_2. Regulated control of substrate availability represents a novel mechanism for regulation of PLC-β activity and for crosstalk between G_q-coupled receptors and receptors that activate the Akt kinase pathway.

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Results

WNK1 Activates TRPC6 Channels

The TRPC6 channel is directly activated by DAG, a product of the PLC-catalyzed hydrolysis of PIP_2 [24]. Inspired by a serendipitous observation that WNK1 knockdown altered G_q signaling, we used TRPC6 as a biosensor to examine the role of WNK1 in PLC-β signaling in native cell membranes. Full-length WNK1 is difficult to purify, and we tested the effect of WNK1 on TRPC6 by intracellular application of a purified fragment of rat WNK1 consisting of residues 1–491 ($WNK1_{1–491}$), which contains the Akt-phosphorylation site, the protein kinase domain and a N-terminal proline-rich domain (Figure 1A). As shown for HEK293 cells in Figure 1B, intracellular $WNK1_{1–491}$ increased TRPC6 current density, peaking in these cells at around 1–2 min and lasting for > 5 min. The half-maximal concentration of $WNK1_{1–491}$ for activation of TRPC6 was ~50 nM (Figure 1C). $WNK1_{1–491}$ also activates TRPC6 in membranes of HeLa cells (see Figure 6) and mouse embryo fibroblasts (see Figures 2C and 2D). TRPC6 was also activated by GTPγS, consistent with G_q-PLC-β signaling to produce DAG, and by the muscarinic agonist carbachol in cells that express the G_q-coupled M3 muscarinic cholinergic receptor (Figure 1B).

Application of $WNK1_{1–491}$ to the cytoplasmic face of detached plasma membranes also activated TRPC6 single channel activity in membranes of cells that express TRPC6 (Figure S1A) but not in membranes from control cells. Thus, the cellular components required for activation of TRPC6 by WNK1 are contained in or are bound to the excised plasma membrane patch. Stimulation of TRPC6 was selectively blocked by antibody against amino acid residues 160–176 of WNK1 (Figure S1B). Purified $WNK1_{1–491}$ carrying a kinase-dead K233M mutation [1, 9] retained full ability to activate TRPC6 (Figure S1C), indicating that kinase activity of WNK1 is dispensable.

Activation of TRPC6 by WNK1 Requires PLC Activity

Several experimental results argue that the activation of TRPC6 by WNK1 is mediated by PLC-β-catalyzed production of the known TRPC6 activator DAG. First, activation of TRPC6 by $WNK1_{1–491}$ was blocked by the PLC inhibitors U73122 (Figure 1D) and edelfosine [25] (Figure 1E). Application of 1-oleoyl-2-acetyl glycerol (OAG), a soluble and membrane-permeant DAG, restored TRPC6 activity in the continued presence of U73122, which excludes effects of U73122 on the channel itself or on other components upstream of PLC. Second, because edelfosine acts slowly [25], we could rescue WNK1 signaling by addition of purified PLC-β1 after endogenous PLC was inhibited (Figure 1E). After edelfosine pretreatment, application of both purified PLC-β1 and $WNK1_{1–491}$ caused robust activation of TRPC6. Application of PLC-β1 without WNK1 had no effect, indicating that both WNK1 and PLC-β1 are required for activation of TRPC6. Third, PLC activity is dependent on cytosolic Ca^{2+} [17, 18], and removal of Ca^{2+} blocked TRPC6 stimulation by $WNK1_{1–491}$ but not direct stimulation by OAG (Figure 1F). Based only on the data above, $WNK1_{1–491}$ might activate TRPC6 by protecting DAG from hydrolysis by DAG lipase or phosphorylation by DAG kinase. However $WNK1_{1–491}$ did not alter the potency with which OAG activates TRPC6 (Figure 1G), indicating that its effect is at the level of PLC. Together, these results provide compelling evidence that WNK1 activates TRPC6 by stimulation of PLC to produce DAG, the proximal activator.

We also measured intracellular Ca^{2+} release triggered by IP_3, another product of PIP_2 hydrolysis, as a read-out for WNK1's effects on PLC activity. Microinjection of anti-WNK1 antibody or knocking down endogenous WNK1 by small interfering RNA (siRNA) reduced the intracellular Ca^{2+} increases in response to carbachol (Figures S1D–F). Endogenous WNK1 is thus required for normal PLC-β signaling in response to stimulation of G_q.

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WNK1 Works in Parallel With G_q to Synergistically Stimulate TRPC6

The PLC-βs are activated by $G\alpha_q$ [17, 18]. GDPβS, which blocks G protein activation, inhibited the stimulation of TRPC6 by $WNK1_{1–491}$ (Figure 2A). GTPγS stimulated the TRPC6 current without added WNK1, and stimulation was blocked by anti-WNK1 antibody (Figure 2B, Figures S2A and S2B). These data suggest both that some G protein is required for WNK1 stimulation of TRPC6 and that endogenous WNK1 is required for TRPC6 stimulation by a G protein. To determine whether WNK1 requires activation of PLC-β by $G_{q/11}$ in cells, we measured TRPC6 regulation by WNK1 in murine $G\alpha_q^{-/-}$-$G\alpha_{11}^{-/-}$ embryonic fibroblasts [26]. In intact $G\alpha_{q/11}$-null cells, $WNK1_{1–491}$ failed to activate TRPC6, and expression of wild type $G\alpha_q$ restored activation by WNK1 (Figure 2C). When the constitutively active Q209L-$G\alpha_q$ mutant was expressed, addition of $WNK1_{1–491}$ further activated TRPC6 (Figures S2C and S2D), which argues that WNK1 and $G\alpha_q$ act in parallel rather than in series to stimulate DAG synthesis by PLC-β.

When WNK1 was depleted in the $G\alpha_{q/11}$-null cells by siRNA, neither $WNK1_{1–491}$ nor $G\alpha_q$-GTPγS alone stimulated TRPC6 substantially, only simultaneous addition of both activators produced major TRPC6 opening (Figure 2D). Moreover, the G_q inhibitor RGS4 blocked activation of TRPC6 by WNK1 in cells and the N128G mutant of RGS4 [27] had no effect (Figures S2E and S2F). In detached patches, inhibition by RGS4 was reduced and delayed by expression of the RGS-insensitive G188S mutant of $G\alpha_q$ [28, 29] (Figures 2E and 2F). Thus, WNK1 and activated $G\alpha_q$ stimulate TRPC6 synergistically.

WNK1 Activity Is Necessary for Activation of TRPC6 by G_q-Coupled Receptors

Intracellular application of anti-WNK1 antibody markedly blunted the activation of TRPC6 by agonist through the expressed M3 muscarinic receptor (Figure 3A), the α_{1A}-, α_{1B}-adrenergic receptors and H1 histaminergic receptor in HEK cells (Figures S3A–C) or through endogenous muscarinic receptor in HeLa cells (Figure S3D). Conversely, increased WNK1 activity potentiated activation of TRPC6 by M3 receptor. Intracellular application of 3 nM $WNK1_{1–491}$, which is too low to activate TRPC6 by itself, approximately doubled the TRPC6 current elicited by M3 muscarinic stimulation (Figure 3B). Thus, some level of endogenous WNK1 activity is necessary for full G_q-PLC-β signaling and additional WNK1 potentiates G_q signaling in cells.

Anti-WNK1 antibody did not significantly inhibit activation of TRPC6 by the G_i-coupled M2 muscarinic receptor (Figure S3E). However, G_i is a less effective activator of PLC-βs [17, 18], and TRPC6 activation by the M2 receptor is much smaller than that by the G_q-coupled receptors (Figure S3E vs Figure 3A). This apparent selectivity of anti-WNK1 antibody for G_q-coupled receptors suggests that WNK1 is needed to support the higher flux of the G_q-PLC-β pathway but is not detectably required for G_i-PLC-β signaling.

While these data are consistent with the stimulation of PLC-β activity by WNK1, we did not find any effect of purified $WNK1_{1–491}$ on the activity of purified PLC-β1, 2 or 3; its regulation by $G\alpha_q$ or Gβγ; or regulation of G_q by M1 muscarinic receptors (Figure S4).

Activation of TRPC6 by WNK1: PI4K-Mediated PIP_2 Synthesis

The interdependence and mutual potentiation of TRPC6 activation by the $G\alpha_q$ and WNK1 pathways suggests that they each increase the activity of PLC-β by distinct mechanisms. $G\alpha_q$ allosterically stimulates the catalytic activity of PLC-β. We therefore asked whether WNK1 acts by increasing the concentration of the PIP_2 substrate. Generation of phosphatidylinositol 4-P (PI4P) by PI4K is believed to be the first step in the synthesis of PIP_2 [30]. Pretreatment of cells with the PI4K inhibitor wortmannin inhibited activation of TRPC6 by $WNK1_{1–491}$ (Figure 4A) as did simultaneous application of an anti-PI4P

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antibody [31] (Figure 4B). Last, intracellular application of PI4KIIIβ activated TRPC6 even in the absence of added WNK1, and pretreatment with wortmannin prevented activation (Figure 4C). PI4P synthesis is thus involved in the stimulation of TRPC6 by WNK1, and WNK1 can be mimicked by addition of a PI4K.

We used the GFP-tagged PH domain of PLC-δ1 and confocal imaging to examine the role of WNK1 in the degradation and re-synthesis of PIP_2 in the plasma membrane. The PLC-δ1-PH domain binds both PIP_2 and IP_3 with relatively high affinity and specificity [32–34]. In cells that express M3 receptor, addition of carbachol decreased GFP-PLC-δ1-PH fluorescence at the plasma membrane, indicating reduced PIP_2, and increased fluorescence in the cytosol, indicating increased IP_3 (Figure 5). Both effects peak ~45 sec after carbachol addition and then decline to pre-stimulus levels, reflecting PLC-β-catalyzed hydrolysis of PIP_2 and its subsequent re-synthesis.

Microinjection of anti-WNK1 antibody both delayed and decreased the recovery of GFP-PLC-δ1-PH fluorescence at the plasma membrane after treatment with carbachol. Instead of the monophasic return to baseline in <3 min observed in control cells, cells treated with anti-WNK1 antibody displayed biphasic recovery of plasma membrane fluorescence that was only ~50% complete after 15 min. The return of cytosolic fluorescence to baseline was also biphasic, with a greatly prolonged and incomplete second phase. These data indicate that anti-WNK1 antibody substantially inhibits re-synthesis of PIP_2 after a burst of PLC activity. Taken together with the effects of the anti-PI4P antibody, they indicate that WNK1 is necessary to maintain cellular PIP_2 synthesis.

WNK1 Potentiates PI4P Synthesis by PI4KIIIα

The PI4KIIIα isozyme is believed to be primarily responsible for re-synthesis of PIP_2 in the plasma membrane during receptor stimulation of PLC [30]. To determine whether WNK1 regulates PI4KIIIα, we studied effects of manipulating WNK1 activity in cells that express recombinant PI4KIIIα, where this isoform is the predominant PI4K activity (~95%) in a light membrane fraction, which includes plasma membranes (Figure 6A). Knockdown of WNK1 by siRNA decreased both PI4K activity and the amount of immunoreactive PI4KIIIα in the light membrane fraction by 35–40% (Figures 6B–E) and increased the abundance of PI4KIIIα in cytosol (Figures 6D and 6E). WNK1 knockdown did not alter the amount of PI4KIIIα in the total post-nuclear supernatant (PNS). These data suggest that WNK1 recruits PI4KIIIα from cytosol to the light membrane fraction, presumably the plasma membrane. Recruitment may be indirect, because we did not detect physical association between PI4KIIIα and purified $WNK1_{1–491}$ in pull-down assays.

WNK1 failed to activate TRPC6 in PI4KIIIα-knockdown cells (Figures 6F and 6G) and expression of PI4KIIIα in the knockdown cells rescued activation by WNK1. These results are consistent with the idea that WNK1 increases PIP_2 synthesis by recruitment of PI4KIIIα to its site of action, and that the consequently increased availability of PIP_2 synergistically enhances G_q-coupled receptor signaling through PLC-β.

IGF1 Potentiates G_q-PLC-β Signaling *via* PI3K and Akt-Mediated Phosphorylation of WNK1

The physiological role of WNK1 in the regulation of G_q-PLC-β signaling was further supported by the finding that IGF1, a known upstream activator of WNK1, enhances the effect of WNK1. Pretreatment with IGF1 in serum-starved cells potentiated the activation of TRPC6 by either carbachol (Figures 7A and 7B) or GTPγS (Figures S5A and S5B), although IGF1 did not alter basal TRPC6 current. Lack of effect on the basal current is probably due to a low basal output from G_q-PLC-β in serum-starved cells, and IGF1 activated TRPC6 in these cells when pre-stimulated by GTPγS (Figure S5C). IGF1 also

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enhanced carbachol-induced intracellular Ca^{2+} transients (Figure S5D), consistent with a final common site of action at PLC-β. IGF1 did not increase the cell-surface abundance of TRPC6 (Figure S5E).

Several observations argue that IGF1 potentiates GPCR-G_q signaling through a PI3K-Akt-WNK1-PI4KIIIα sequence. First, wortmannin, at a low concentration selective for PI3K (20 nM), blocked the effect of IGF1 on G_q signaling. Knockdown of WNK1 also blocked the ability of IGF1 to potentiate carbachol's effect (Figure 7C). Expression of wild type WNK1, but not the T58A mutant WNK1, restored the ability of IGF1 to enhance carbachol activation of TRPC6 in WNK1-knockdown cells (Figure 7D), indicating that the synergistic effect of IGF1 and M3 muscarinic stimulation depends on Akt-mediated phosphorylation of WNK1 at T58. Note that both wild type WNK1 and the T58A mutant can support carbachol activation of TRPC6 in WNK1-knockdown cells. Thus, T58-phosphorylation is not required for WNK1 amplification of G_q-PLC-β signaling, but is indispensable for its potentiation by IGF1.

Knockdown of PI4KIIIα also prevented enhancement of carbachol activation of TRPC6 and intracellular Ca^{2+} transients by IGF1 (Figure 7E, Figure S5F). These results argue that PI3K-dependent activation of Akt by IGF1 leads to phosphorylation of WNK1 and enhances the ability of WNK1 to synergistically regulate PLC-β signaling by promoting PIP_2 synthesis.

Discussion

Our data establish three new and distinct but inter-related signaling mechanisms: the mutual dependence and synergistic interaction between the G_q-PLC-β and Akt-WNK1 signaling pathways; the regulation of PLC-β activity by modulating the availability of its substrate; and the regulation of PI4KIIIα by WNK1 to control the concentration of PIP_2-derived second messengers.

As summarized in Figure 7F, blocking WNK1 activity markedly inhibits PLC-β signaling by G_q-coupled receptors and, conversely, inhibiting G_q blocks PLC-β signaling by WNK1. Inhibition could be exerted by several approaches and at several steps in each pathway, both in cells and in detached membrane patches. In each case, replenishment of the inhibited or depleted species, or stimulation of a downstream component, restored normal activities of both WNK1 and G_q-PLC-β. Importantly, WNK1 can elicit PLC signals without added GPCR agonist, and WNK1 synergistically amplifies the stimulation of PLC-β by G_q.

In contrast to G proteins, WNK1 increases PLC-β activity by increasing the supply of the PIP_2 substrate. Because the concentration of PIP_2 in the plasma membrane is below K_m [22, 23], PLC-β displays first-order substrate kinetics and increase in available PIP_2 linearly amplifies PLC-β output. Note that WNK1 only modulates G_q signaling *via* PLC-β. Other outputs, such as stimulation of a rho GEF [36], are not involved. WNK1 thus alters the spectrum of G_q outputs as well as its amplitude.

WNK1 promotes synthesis of PIP_2 by somehow activating PI4KIIIα (Figures 6 and 7F). PI4KIIIα is important for the re-synthesis of PIP_2 following hydrolysis by receptor-stimulated PLC [30], but how the activity of PI4KIIIα is regulated is unknown. The amount of PI4KIIIα is decreased in membranes from WNK1 knockdown cells, suggesting that regulation of PI4KIIIα by WNK1 may involve its redistribution between plasma membrane and intracellular pools. However, WNK1 must do more than just recruit PI4KIIIα to the plasma membrane because it remains necessary for PLC-β signaling in detached patches. We have no evidence that WNK1 either binds or phosphorylates PI4KIIIα, and additional factors may be involved.

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Regardless, our results indicate that the activity of PI4KIIIα is regulated and that its regulation by WNK1 is critical for PLC-β signaling by G_q-coupled receptors. The basal activity of WNK1 provides adequate tonic stimulation of PI4KIIIα to provide PIP_2 for normal PLC-β signaling. However, additional WNK1 markedly amplifies signaling by G_q-coupled receptor even at a concentration that does not by itself activate TRPC6 (Figure 3B). In addition, PI3K-activating growth factors such as IGF1 activate WNK1 to further potentiate G_q-PLC-β signaling, probably *via* Akt-mediated phosphorylation of WNK1 at T58 (Figure 7). These results suggest that WNK1 is an important physiological mediator of the Akt kinase signaling pathway and coordinates Akt signaling with the G_q-PLC-β signaling pathway.

This action of WNK1 represents the first known instance of acute control of PIP_2 availability as a mechanism of regulation of cellular DAG and IP_3 concentrations without concurrent stimulation of the intrinsic activity of PLC. Application of WNK1 alone stimulates the production of DAG measured using the TRPC6 biosensor by an amount comparable to that initiated by GTPγS (Figure 1B). Some low output of PLC-β, evidently dependent on basal input from $G\alpha_q$, is adequate to support this WNK1 signaling. By promoting PIP_2 synthesis, WNK1 can also acutely modulate the amplitude of receptor-stimulated PLC-β signaling and/or its duration. Inhibition of endogenous WNK1 by anti-WNK1 antibody blocks both the DAG and IP_3 limbs of PLC-β signaling stimulated by multiple G_q-coupled receptors by ≥50% (Figure 3A and Figures S1D–F and S3). Acute stimulation of WNK1 activity by IGF1 potentiates PLC-β signaling by ~2-fold (Figures 7A and 7B). WNK1 thus controls a major mode of desensitization of G_q signaling in addition to modulating acute responses. PIP_2 itself is a signaling molecule that regulates many plasma membrane targets [37]. Increasing PIP_2 synthesis by WNK1 is thus an important mechanism for increasing cellular DAG and IP_3 concentrations without causing untoward effects of decreases in membrane PIP_2 on other targets.

PLC-β enzymes function as co-incidence detectors that synergistically integrate multiple upstream inputs including $G\alpha_q$, Gβγ, Ca^{2+}, and Rac [35, 38, 39]. These mechanisms of synergism are results of combined super-additive stimulation of the catalytic activity of PLC-β. In contrast, synergism between WNK1 and $G\alpha_q$ depends on the ability of WNK1 to enhance substrate supply for PLC-β. This system is analogous to the interaction between PLCs and inositol phosphate kinases in which PLC provides the IP_3 starting material that the cell-type-specific kinases then phosphorylate to the appropriate inositol polyphosphates [40].

The interactive control of PLC-β by WNK and G_q may have important implications in the pathogenesis of hypertension in patients with PHA2 that is caused by mutations of *WNK1* that increase WNK1 expression [2]. $G_{q/11}$-mediated PLC-β signaling plays a central role in the regulation of vascular tone by virtually all vasoactive hormones [41], and potentiation of PLC-β signaling by WNK1 would promote vasoconstriction and cause hypertension. The role of WNK1 in the regulation of vascular tone is supported by the report that mice with heterozygous inactivation of *Wnk1* display reduced blood pressure without renal Na^+ wasting and hypovolemia [42]. Finally, the similarity between the phenotypes of WNK1-knockout and $G\alpha_q/G\alpha_{11}$-knockout mice (both lethal at embryonic day ~10.5, with cardiac defects) supports the idea that interaction between the WNK1 and $G\alpha_{q/11}$ pathways is important for embryonic development [10, 26].

In conclusion, WNK1 potentiates G_q-PLC-β signaling by stimulating the activity of PI4KIIIα to increase synthesis of PIP_2. Phosphorylation of WNK1 by Akt kinase increases its activity in this pathway, and allows tyrosine kinase receptors or other activators of the Akt kinase cascade to regulate G protein-mediated PLC-β signaling. The extent and

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physiological role of WNK1 in the regulation of PLC-β signaling will probably differ among specialized cells. Our understanding of these interactions should expand as we learn more ways in which WNK1 activity is regulated.

Experimental Procedures

Detailed experimental procedures are in Supplementary Information. Human embryonic kidney (HEK)-293, HeLa, and mouse embryonic fibroblasts were cultured and co-transfected with cDNAs for TRPC6-Flag with or without human M3 muscarinic receptor [43]. Ruptured whole-cell, cell-attached, and inside-out excised patch-clamp recording were performed as described [9, 43]. Intracellular Ca^{2+} transients were examined using ratiometric fluorescent Ca^{2+} images of fura-2-loaded cells. PIP_2 and IP_3 were localized according to fluorescence of a GFP-tagged PLC-δ1-PH domain by confocal microscopy [34]. PLC activity was measured by monitoring hydrolysis of $[^3H]PIP_2$ on large unilamellar phospholipid vesicles [22, 35]. PI4KIIIα activity from HeLa cells was measured by phosphorylation of phosphatidylinositol (PI) in PI/Triton X-100-mixed micelles [44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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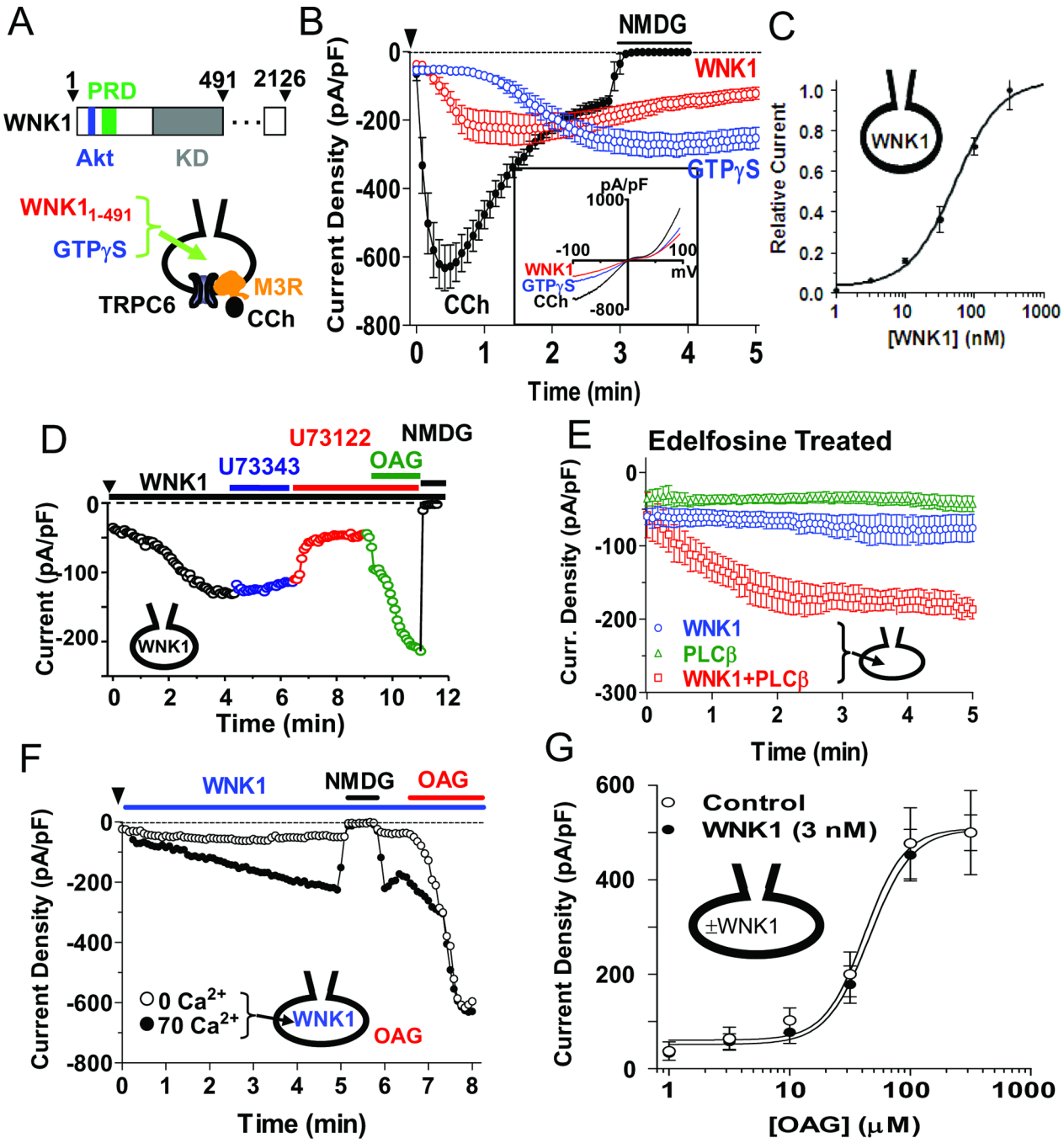

Figure 1.

Activation of TRPC6 by WNK1 requires PLC activity and is independent of WNK1 kinase activity. (A) Top: Domain structure of rat WNK1. Akt, its phosphorylation site; PRD, proline-rich domain; KD, kinase domain. Bottom: whole-cell patch-clamp recording of TRPC6. CCh: carbachol (100 μM); M3R: M3 muscarinic cholinergic receptor. Intracellular free [Ca^{2+}] was 70 nM except as indicated in panel F. (B) Activation of TRPC6 by CCh via M3R, GTPγS or $WNK1_{1–491}$. HEK cells were transfected with TRPC6 with or without M3R. Data points (circles and error bars) are means ± SEM of inward current density (pA/pF at −100 mV; n = 5 for each). At the end of each recording, extracellular Na^{+} was replaced by the TRPC6-impermeable cation N-methyl-D-glucamine (NMDG) to gauge the

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background current. Inset shows current-voltage (I–V) relationships of activated TRPC6 currents. (C) Concentration-response curve for activation of TRPC6 by $WNK1_{1–491}$. (D) Effect of PLC inhibitor U73122 or inactive analog U73343 (2.5 μM each) on $WNK1_{1–491}$ activation of TRPC6. Inward current at −100 mV from a representative experiment. Similar results were observed in 6 independent experiments. (E) Cells were preincubated with 15 μM edelfosine for 1 hr and washed before recording. TRPC6 currents in response to 100 nM $WNK1_{1–491}$, 5 nM PLC-β1 or both were measured. (F) TRPC6 currents in response to $WNK1_{1–491}$ under 0 or 70 nM intracellular calcium. OAG was applied as indicated. (G) TRPC6 currents were measured in cells with or without intracellular application of 3 nM $WNK1_{1–491}$. OAG was applied to the extracellular solution at indicated concentrations. Although this concentration of $WNK1_{1–491}$ by itself does not activate TRPC6, it potentiated M3R activation of the channel (see Figure 3B). (see also Figure S1).

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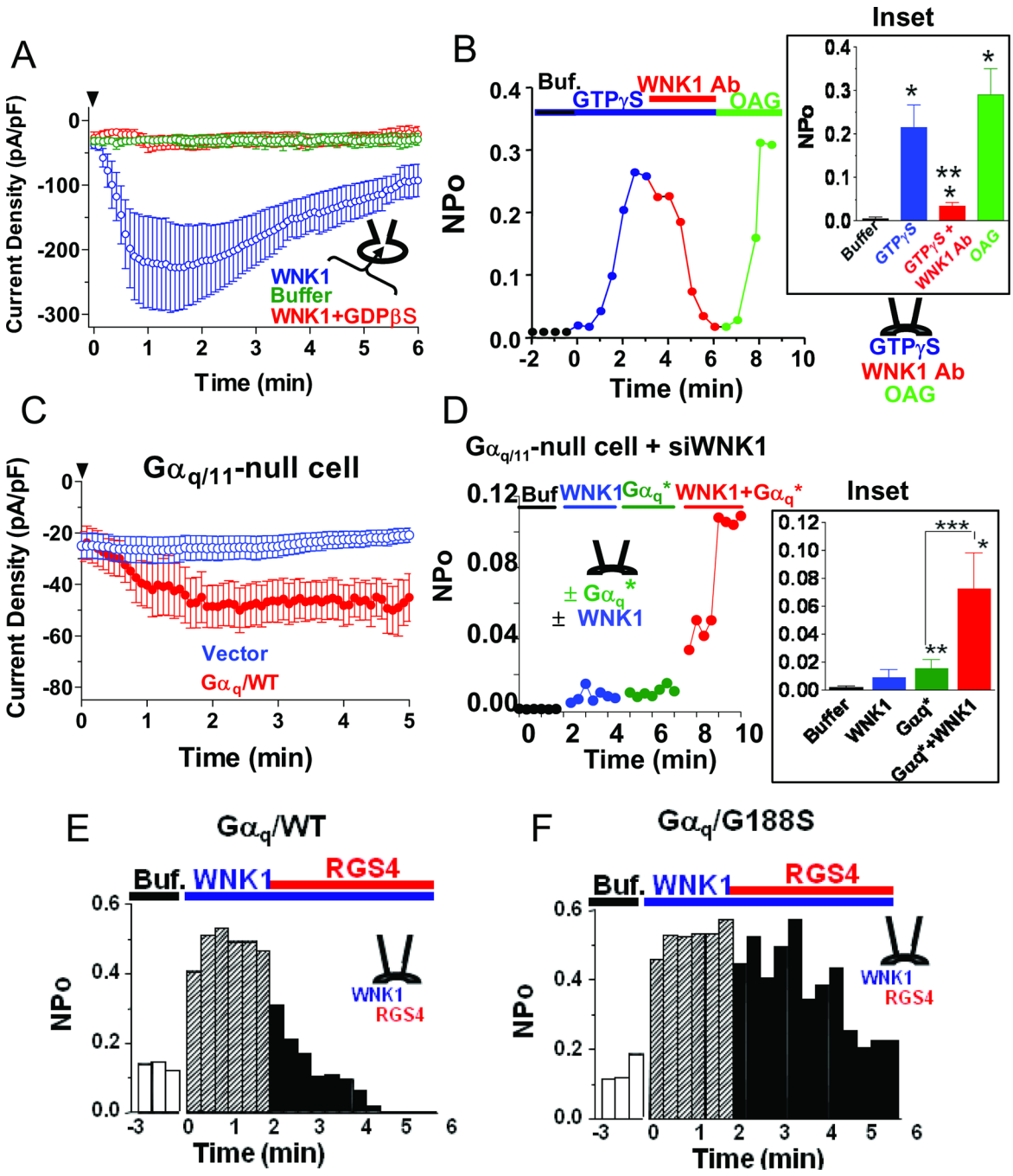

Figure 2.

Role of G_q in the activation of TRPC6 by WNK1. (A) GDPβS (0.2 mM) inhibits WNK1 activation of TRPC6. (B) Anti-WNK1 antibody inhibits TRPC6 activated by GTPγS. TRPC6 single channel activity was recorded from inside-out patches from TRPC6-expressing HEK cells. Buffer, GTPγS, anti-WNK1 Ab and/or OAG was applied to the cytoplasmic face as indicated. Each data point is NPo (number × open probability) averaged over 30 s. Inset shows mean ± SEM of peak open probability. *, $p < 0.01$ vs "buffer". **, $p < 0.01$ "GTPγS" vs "GTPγS+anti-WNK1 antibody". (C) Whole-cell WNK1-activated TRPC6 currents were measured in $G\alpha_{q/11}$-null fibroblasts transfected with wild type $G\alpha_q$ ($G\alpha_q$/WT) or empty vector. (D) Single channel activity was measured in inside-out patches

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from TRPC6-expressing $G\alpha_{q/11}$-null fibroblasts that have endogenous WNK1 depleted by siRNA. Data show responses to GTPγS-preactivated $G\alpha_q$ ("$G\alpha_q$*") and/or WNK1 applied to the cytoplasmic face. Holding membrane potential is −80 mV. Inset shows mean ± SEM of peak open probability. *, $p < 0.01$ vs "buffer". **, $p < 0.05$ vs "buffer". ***, $p < 0.02$ between indicated groups. (E, F) Single channel activity was recorded in inside-out patches from HEK cells expressing both TRPC6 and wild type $G\alpha_q$ ($G\alpha_q$/WT) (panel E) or G188S-mutant $G\alpha_q$ ($G\alpha_q$/G188S) (panel F). Buffer, $WNK1_{1–491}$ and/or RGS4 (0.5 μM) was applied to the cytoplasmic face as indicated. NPo values were averaged every 20 s. There was a 2 min time gap between application of buffer and WNK1. (see also Figure S2).

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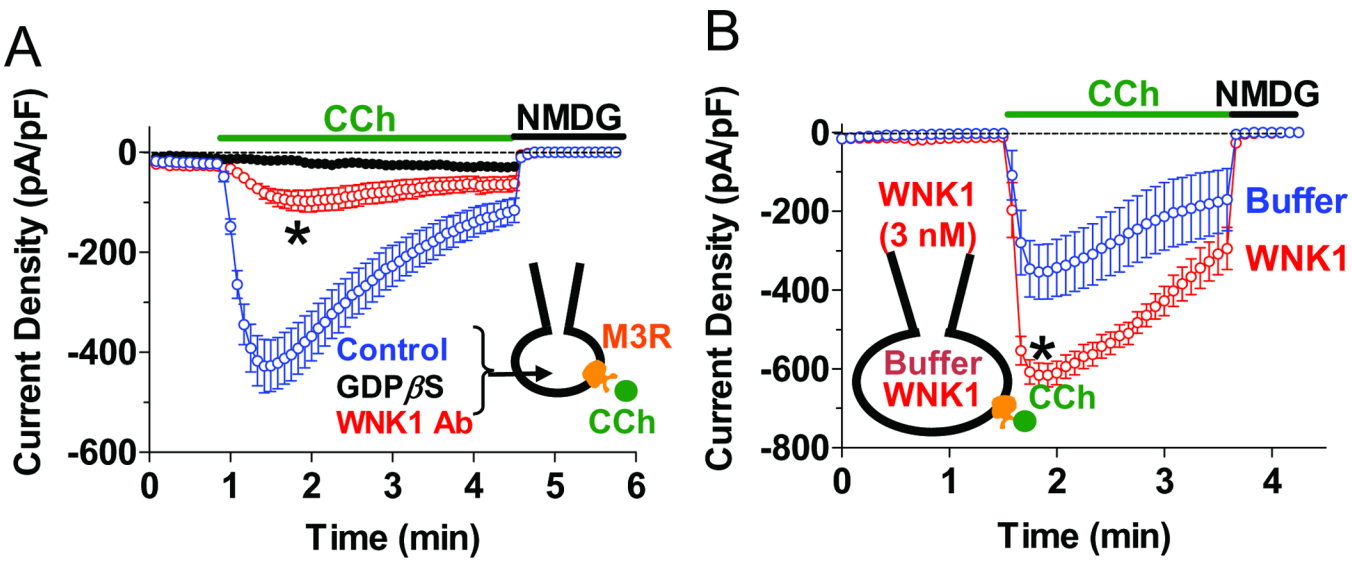

Figure 3.

Role of WNK1 in activation of TRPC6 by G_q-coupled receptor. (A) Whole-cell inward TRPC6 current density in response to CCh with anti-WNK1 Ab, GDPβS or control buffer applied via patch pipettes. *, $p < 0.01$, WNK1-Ab vs control. (B) Whole-cell CCh-induced TRPC6 currents were recorded in the presence of control buffer or $WNK1_{1–491}$ (3 nM), a concentration that does not appreciably stimulate TRPC6 by itself. *, $p < 0.01$, WNK1 vs buffer. (see also Figure S3).

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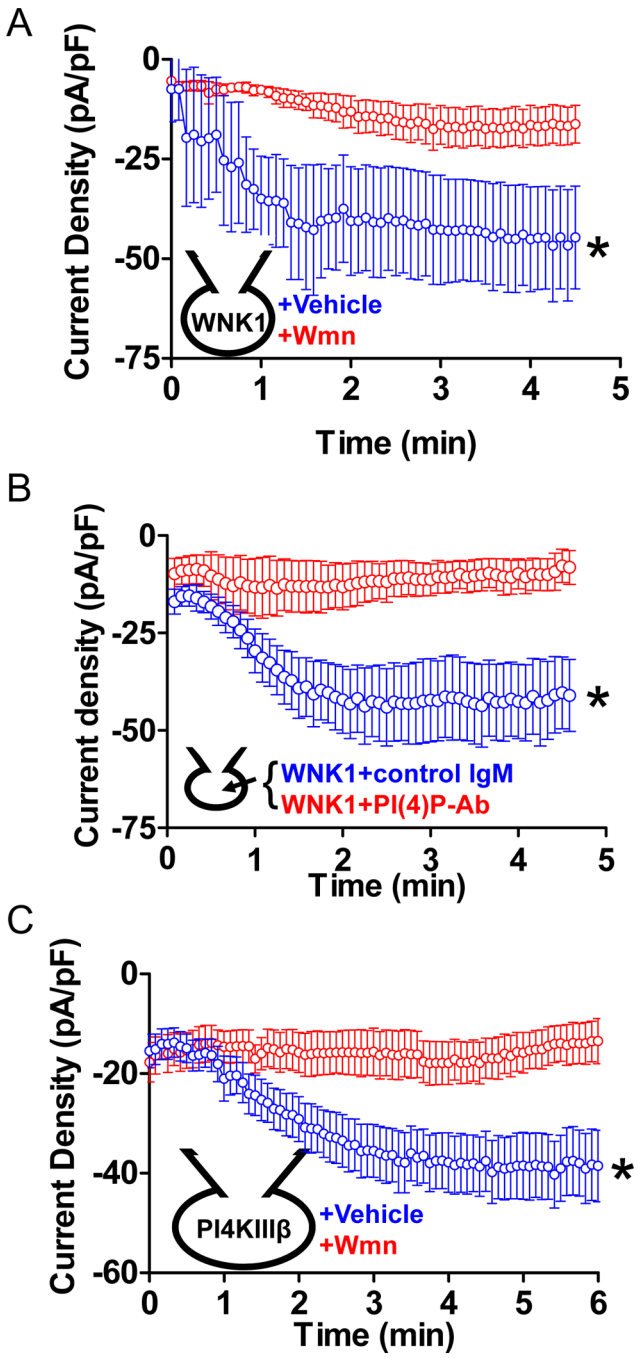

Figure 4.

Role of PI4K in the activation of TRPC6 by WNK1. (A) Effect of wortmannin (Wmn) on whole-cell TRPC6 currents activated by $WNK1_{1–491}$. Cells were pretreated with 10 μM wortmannin or vehicle (DMSO) for 10 min before recording. *, $p < 0.01$, vehicle vs wortmannin. (B) Whole-cell TRPC6 currents in response to $WNK1_{1–491}$ co-applied with monoclonal anti-PI4P antibody or control mouse IgM. *, $p < 0.01$, control IgM vs PI(4)PAb. (C) Whole-cell TRPC6 current in response to intracellular delivery of 100 nM PI4KIIIβ preincubated with or without 10 μM wortmannin for 10 min. *, $p < 0.01$, vehicle vs wortmannin. Note that both type IIIα and IIIβ PI4K can generate PI4P for synthesis of PIP_2 in the membrane. Due to difficulties in purification of PI4KIIIα, we used only PI4KIIIβ for

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experiments here. In all panels, data point is mean ± SEM of inward current density, n= 5–9 for each condition. (see also Figure S4).

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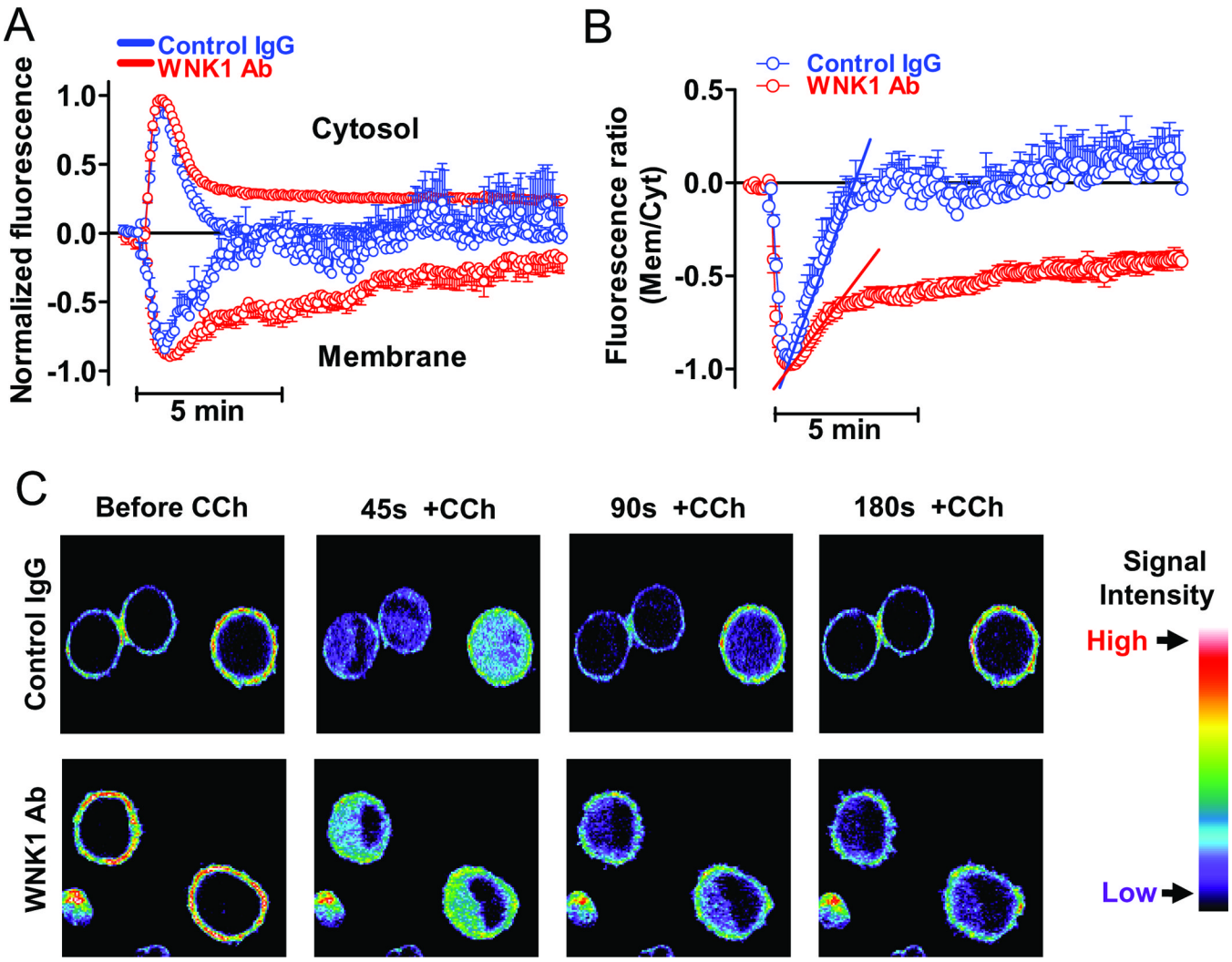

Figure 5.

Anti-WNK1 antibody delays and inhibits plasma membrane PIP_2 re-synthesis following receptor-stimulated hydrolysis. HeLa cells transfected with M3R and GFP-tagged PLC-δ1-PH domain were microinjected with control IgG or anti-WNK1 antibody and stimulated by 1 μM CCh. (A) Changes in fluorescence intensity at the plasma membrane (decrease) and cytosol (increase) in response to carbachol were normalized by setting maximal change to 1.0. Data are means ± SEM of 18 and 20 cells (7 separate experiments) that were microinjected with control rabbit IgG or anti-WNK1 antibody, respectively. (B) Ratio of fluorescence intensity in the plasma membrane to that in cytosol from panel A. Lines indicate slopes of the initial recovery rates (0.032 ± 0.002 and 0.014 ± 0.002 for control IgG and anti-WNK1 antibody, respectively; $p<0.01$). (C) Representative confocal images (obtained across the middle of cells) from one of 7 experiments showing fluorescence at the plasma membrane and cytosol before addition of CCh and at 45 s (peak), 90 s and 180 s after addition of CCh. Signal intensity is shown by pseudo-color.

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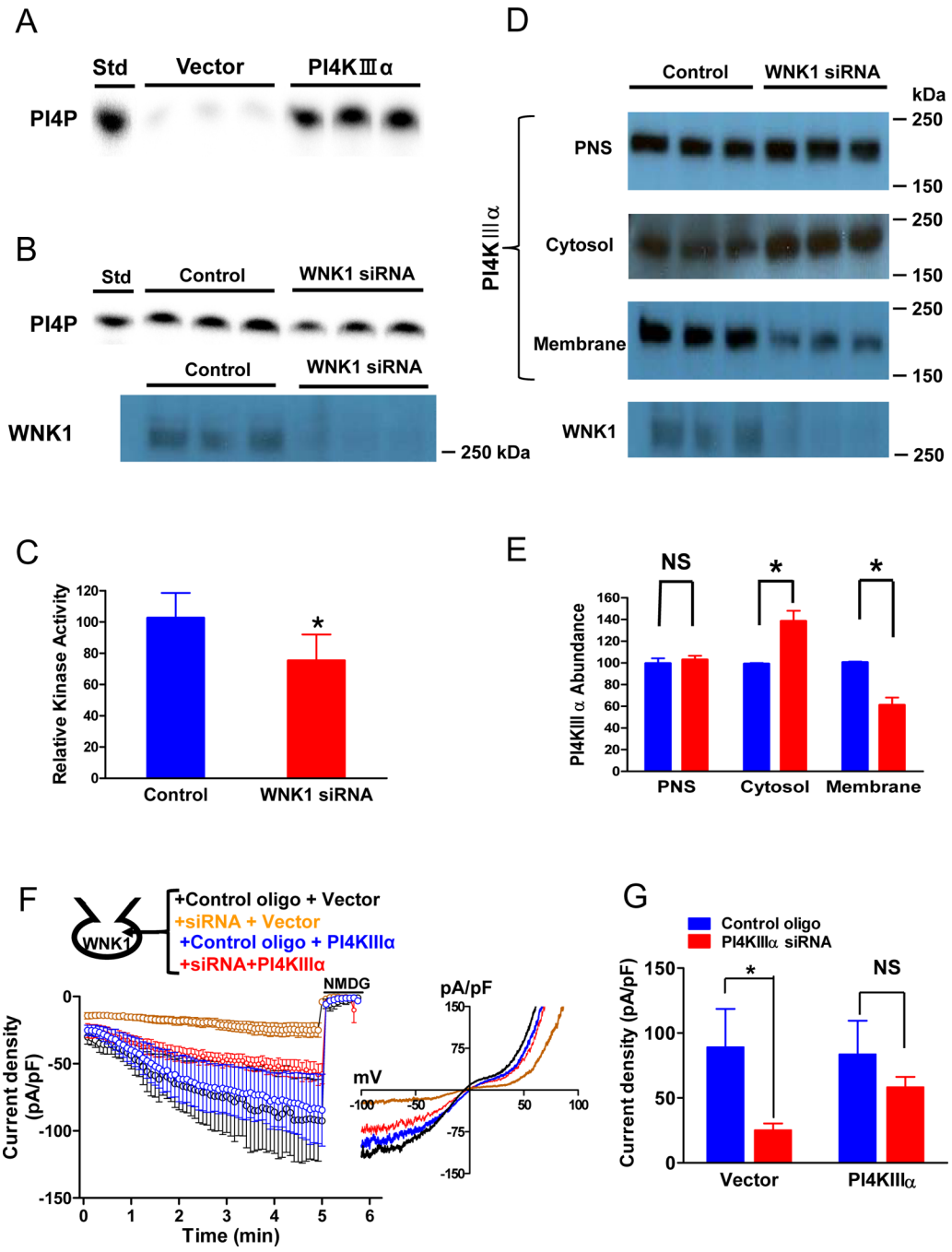

Figure 6.

Role of WNK1 in the production of PI4P by expressed PI4KIIIα. (A) Chromatogram showing PI4K activity in membranes prepared from HeLa cells transfected with vector or cDNA for PI4KIIIα. Std: PI4P standard. (B) Chromatogram showing PI4P production by membrane-associated recombinant PI4KIIIα from cells that were co-transfected with control oligonucleotide or WNK1 siRNA. Relative knockdown of WNK1 is shown in the Western blot. (C) Mean ± SEM of PI4P production from 5 separate experiments, each with triplicate samples, as shown in panel B. *, $p < 0.01$ control versus knockdown. (D) Abundance of PI4KIIIα in the post-nuclear supernatant (PNS), cytosol, and membrane fractions from control and WNK1 siRNA-transfected HeLa cells. A western blot at bottom shows relative

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WNK1 knockdown. (E) Mean ± SEM (5 separate experiments) of PI4KIIIα abundance in the post-nuclear supernatant (PNS), cytosol, and membrane fraction in control (blue) and WNK1 knockdown cell (red), shown relative to the control. *, $p < 0.05$ between indicated. (F) Whole-cell TRPC6 currents stimulated by $WNK1_{1–491}$ in cells co-transfected with control oligonucleotide or siRNA for human PI4KIIIα, with either empty vector or plasmid that encodes rat PI4KIIIα. (G) Peak WNK1-induced inward current density from panel F. *, $p < 0.01$ between indicated.

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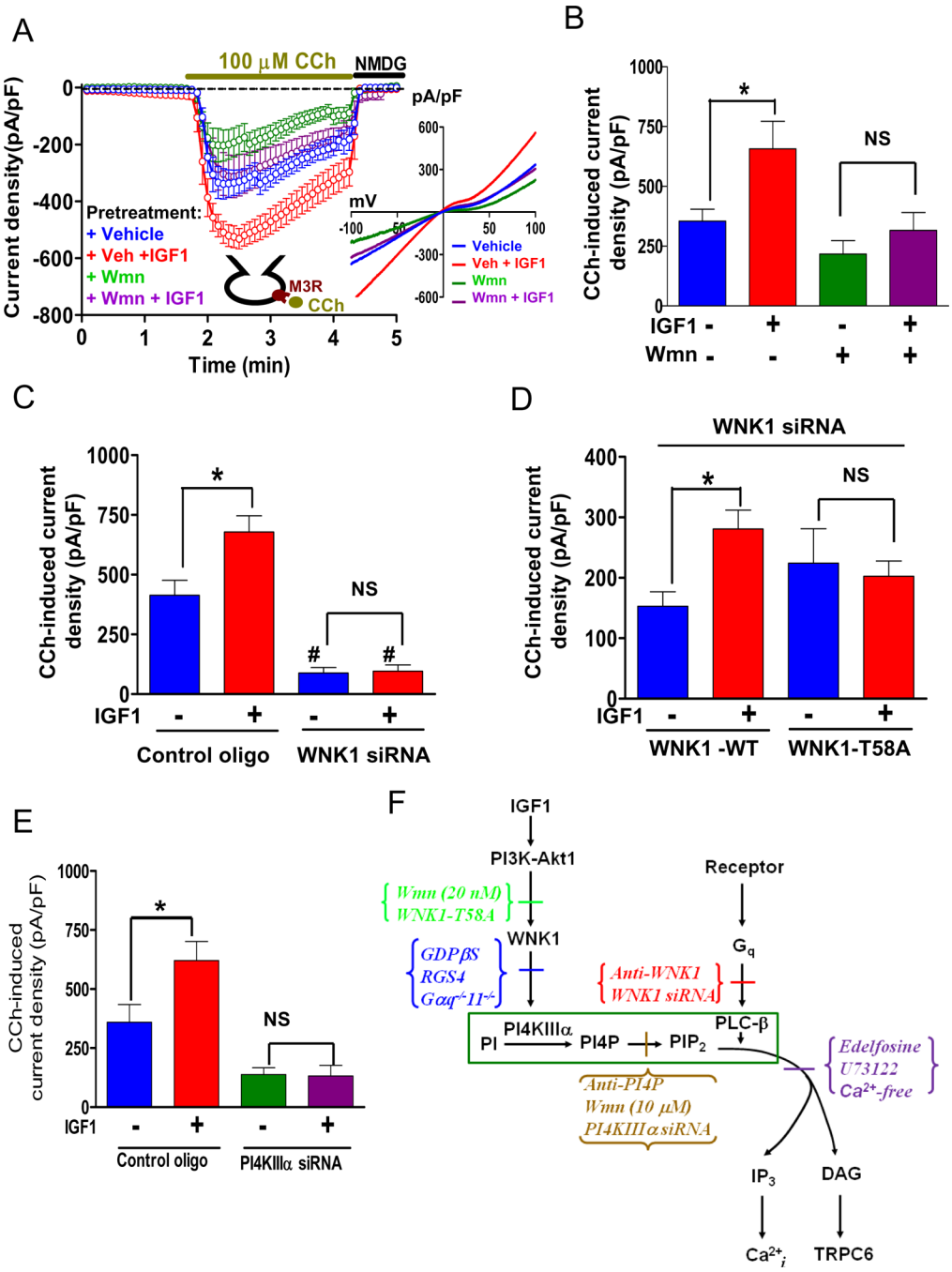

Figure 7.

IGF1 enhances G_q-coupled receptor activation of TRPC6 *via* WNK1. (A) Cells co-expressing TRPC6 and M3R were serum-starved for 18 h and incubated for 10 min with 100 ng/ml IGF1, 20 nM wortmannin, both or neither before ruptured whole-cell recording. Carbachol was applied to the bath where shown. (B) Peak CCh-induced current density from panel A. *, $p < 0.01$ between indicated groups. (C) Peak CCh-induced inward current density in cells transfected with control oligonucleotides or WNK1 siRNA and with or without IGF1 pretreatment. *, $p < 0.01$ between indicated groups. #, $p < 0.01$ vs cells transfected with control oligo and without IGF1 pretreatment. (D) Peak CCh-induced inward current density in cells transfected with WNK1 siRNA and cDNA for either wild type

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WNK1 or T58A mutant WNK1, and with or without IGF1 pretreatment. *, $p < 0.01$ between indicated. (E) Peak CCh-activated TRPC6 currents in cells transfected with control oligonucleotides or PI4KIIIα siRNA and with or without IGF1 pretreatment. *, $p < 0.01$ between indicated. (F) Interaction of WNK1, G_q-PLC-β and PI4KIIIα signaling pathways. Components of the pathways are shown in black. Inhibitors are color-coded per their biochemical sites of action and placed where their inhibitory effects on cellular signaling were noted in the experiments. Pathways are indicated by black arrows. The experimental results indicate that the point at which G_q and WNK1 interacts is at the level of PLC activity (marked by dark green box). (see also Figure S5).