

## Full-length article

## Binding of PLC $\delta_1$ PH-GFP to PtdIns(4,5)P $_2$ prevents inhibition of phospholipase C-mediated hydrolysis of PtdIns(4,5)P $_2$ by neomycin<sup>1</sup>

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### Key words

phosphatidylinositol 4,5-bisphosphate; neomycin; phospholipase C; pleckstrin homology domains; competitive binding; acetylcholine; green fluorescent proteins

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### Abstract

**Aim:** To investigate the effects of the pleckstrin homology (PH) domain of phospholipase C $\delta_1$  (PLC $\delta_1$ PH) on inhibition of phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P $_2$ ] by neomycin. **Methods:** A fusion construct of green fluorescent protein (GFP) and PLC $\delta_1$ PH (PLC $\delta_1$ PH-GFP), which is known to bind PtdIns(4,5)P $_2$  specifically, together with laser-scanning confocal microscopy, was used to trace PtdIns(4,5)P $_2$  translocation. **Results:** Stimulation of the type 1 muscarinic receptor and the bradykinin 2 receptor induced a reversible PLC $\delta_1$ PH-GFP translocation from the membrane to the cytosol in COS-7 cells. PLC inhibitor U73122 blocked the translocation. Wortmannin, a known PtdIns kinase inhibitor, did not affect the translocation induced by ACh, but blocked recovery after translocation, indicating that PtdIns(4,5)P $_2$  hydrolysis occurs through receptor-mediated PLC activation. Neomycin, a commonly used phospholipase C blocker, failed to block the receptor-induced PLC $\delta_1$ PH-GFP translocation, indicating that neomycin is unable to block PLC-mediated PtdIns(4,5)P $_2$  hydrolysis. However, in the absence of PLC $\delta_1$ PH-GFP expression, neomycin abolished the receptor-induced hydrolysis of PtdIns(4,5)P $_2$  by PLC. **Conclusion:** Although PLC $\delta_1$ PH and neomycin bind to PtdIns(4,5)P $_2$  in a similar way, they have distinct effects on receptor-mediated activation of PLC and PtdIns(4,5)P $_2$  hydrolysis.

### Introduction

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P $_2$ ], a minor phospholipid component of the plasma membrane, is a key regulator of several cellular processes, and has become the focus of research on intracellular signal transduction. PtdIns(4,5)P $_2$  is a precursor of important second messengers, such as the diffusible InsP $_3$ , which regulates Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores, and the protein kinase C activator, diacylglycerol<sup>[1,2]</sup>. PtdIns(4,5)P $_2$  is also phosphorylated by class I PtdIns 3-kinases to form PtdIns(3,4,5)P $_3$ , which controls membrane recruitment and the functions of several important signaling proteins<sup>[3]</sup>. PtdIns(4,5)P $_2$  itself is a regulator of a great variety of target molecules, including ion channels<sup>[4,5]</sup> and several proteins that regulate actin polymerization and the cytoskeleton<sup>[6]</sup>, providing a link between the plasma membrane and the cortical

cytoskeleton<sup>[7]</sup>. PtdIns(4,5)P $_2$  has also been implicated in several forms of membrane remodeling events, including the fusion of secretory vesicles with the plasma membrane<sup>[8]</sup>, clathrin-mediated endocytosis<sup>[9]</sup>, and membrane recovery by endocytosis during neurotransmitter release<sup>[10]</sup>. Such diverse functions rely upon interaction of the lipid with a large number of regulator molecules.

Pleckstrin homology (PH) domains have been described in a large number of signaling proteins, and they show remarkable specificity in recognizing various forms of inositides<sup>[11]</sup>. The PH domain of phospholipase C $\delta_1$  (PLC $\delta_1$ PH) binds with high affinity and selectivity to PtdIns(4,5)P $_2$ <sup>[12]</sup>. Recently, a fusion construct of PLC $\delta_1$ PH with enhanced green fluorescent protein (GFP) (PLC $\delta_1$ PH-GFP) was developed as a probe to visualize PtdIns(4,5)P $_2$  in single cells because it binds to PtdIns(4,5)P $_2$  within the plasma and translocates to the cytoplasm after receptor stimulation. Subsequently,

when PtdIns(4,5)P<sub>2</sub> is resynthesized, fluorescence returns to the membrane<sup>[13]</sup>.

It has been demonstrated that neomycin, an aminoglycoside antibiotic with a large positive charge (about +4.5), binds with high affinity to PtdIns(4,5)P<sub>2</sub><sup>[14]</sup>. Later studies also showed that neomycin bound to and neutralized the negative charge of PtdIns(4,5)P<sub>2</sub><sup>[15]</sup>.

Phospholipase C (PLC)-induced PtdIns(4,5)P<sub>2</sub> hydrolysis is an important cell signaling mechanism. Many membrane receptors couple to PLC, and thus regulate PtdIns(4,5)P<sub>2</sub> turnover and subsequent downstream cell signaling<sup>[16]</sup>. A few PLC modulators have been developed and they play an important role in understanding the cell signaling process involving PLC and PtdIns(4,5)P<sub>2</sub>. Neomycin has long been used as a blocker of PLC, although it actually binds to PtdIns(4,5)P<sub>2</sub> and presumably prevents PtdIns(4,5)P<sub>2</sub> from hydrolysis by PLC<sup>[17]</sup>. Previous studies have demonstrated that both PLC<sub>δ1</sub>PH and neomycin bind PtdIns(4,5)P<sub>2</sub> through an electrostatic interaction<sup>[12,14]</sup>. This similar nature of interaction would indicate a similar consequence for PtdIns(4,5)P<sub>2</sub> hydrolysis by PLC. However, in the present study, we demonstrate that although both PLC<sub>δ1</sub>PH and neomycin bind to PtdIns(4,5)P<sub>2</sub>, only neomycin blocks PtdIns(4,5)P<sub>2</sub> hydrolysis by PLC activation.

## Materials and methods

**Reagents and plasmids** Acetylcholine (ACh), bradykinin (BK), wortmannin, neomycin and Fluo 3-AM, the calcium indicators, were purchased from Sigma-Aldrich (St Louis, MO, USA). ACh, BK and neomycin were dissolved in distilled water. U73122 was purchased from Calbiochem (San Diego, CA, USA). U73122 and wortmannin were prepared as stock solutions in dimethylsulfoxide (Me<sub>2</sub>SO), with a final concentration of Me<sub>2</sub>SO of 0.1%. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were products of Hyclone (Logan, UT, USA). COS-7 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. cDNA from the type 1 muscarinic (M<sub>1</sub>) receptor (M<sub>1</sub>R) and bradykinin 2 receptor (BK<sub>2</sub>R), pEGFP-N1 (GFP) and the PLC<sub>δ1</sub>PH construct with GFP (PLC<sub>δ1</sub>PH-GFP) were gifts from Prof DE LOGOTHETIS (Mount Sinai Medical School, NY, USA). Red fluorescent protein (pDsRed-Express-C1, pDsRed) was purchased from Clontech (Mountain View, CA, USA). All other chemicals were of high performance liquid chromatography or analytical grade.

**Cell culture and transfection** COS-7 cells were seeded in 24-well plates on 12-mm glass coverslips, and cultured in 0.3 mL of DMEM supplemented with 10% (*v/v*) FBS, 100 µg/mL streptomycin, and 100 U/mL of penicillin at 5% CO<sub>2</sub>

and 37 °C. When they were 60%–70% confluent, the cells were transiently transfected with DNA constructs for 8 h using calcium phosphate precipitate, with 1 µg of DNA and equal proportions for each kind of plasmid per well. Following transfection, cells were incubated in 10% FBS DMEM for 12–48 h. For fluorescence detection, cells were washed twice with a modified Krebs-Ringer buffer containing (in mmol/L): 120 NaCl, 4.7 KCl, 0.7 MgSO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, 10 glucose, with 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) added (pH 7.4). The coverslips were placed into a flow-through chamber and mounted on an inverted microscope.

**Confocal microscopy and image analysis** For confocal imaging, a Leica (Wetzlar, Germany) DM-IRBE inverted microscope with a 20× objective (numerical aperture 0.7) and fitted with a TCS-SP2 scanhead was used. Excitation of PLC<sub>δ1</sub>PH-GFP and Fluo 3-AM was achieved with a 488 nm argon ion laserline, and emissions were collected at 500–565 nm. pDsRed fluorescence was visualized with excitation at 543 nm and a 570–600 nm emission filter. For translocation studies, a series of confocal images were taken at 3–10 s intervals and stored on disk. Determination of the ratio of membrane to cytosolic fluorescence was carried out by assigning regions of interest for membrane and cytosol. TCS-SP2 confocal software (Leica) was used to analyze data off-line.

**Measurement of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) of single cells** Cells were seeded onto sterile 12-mm borosilicate coverslips in 35-mm Petri dishes, and incubated with 5 mmol/L fluo 3-AM at 37 °C for 45 min. After loading, cells were washed twice and maintained in modified Krebs-Ringer buffer until assay. [Ca<sup>2+</sup>]<sub>i</sub> changes were represented by relative fluorescence intensity calculated by using the equation  $\Delta F/F_0$ , where  $\Delta F$  and  $F_0$  are the change in fluorescence intensity before and after treatment, and the initial fluorescence intensity, respectively<sup>[18,19]</sup>.

**Statistics** Data were analyzed by using the Chi-square test.  $P < 0.05$  was considered to be a statistically significant difference. All data shown are the mean value of at least 5 experiments and are expressed as mean ± SD.

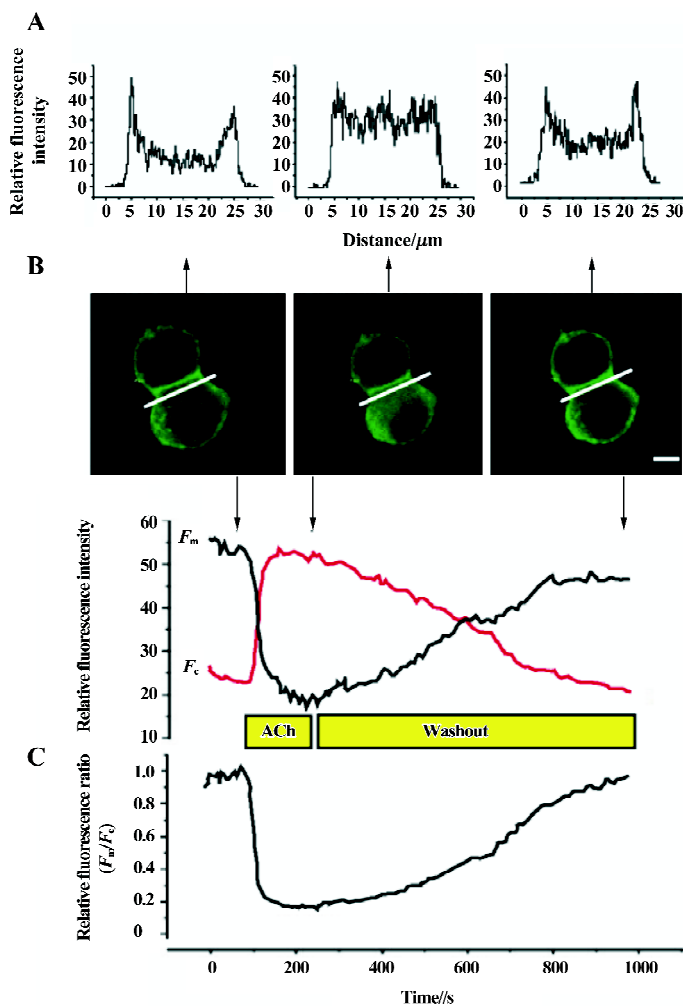
## Results

**Activation of M<sub>1</sub>R and BK<sub>2</sub>R induced PtdIns(4,5)P<sub>2</sub> hydrolysis and a reversible translocation of PLC<sub>δ1</sub>PH-GFP** Both M<sub>1</sub>R, and PLC<sub>δ1</sub>PH-GFP or both BK<sub>2</sub> and PLC<sub>δ1</sub>PH-GFP were expressed in COS-7 cells. To follow the localization of PLC<sub>δ1</sub>PH-GFP within intact cells, we used GFP as a control. In unstimulated cells, expressed GFP was found to be cytosolic and also present in the nucleus (data not shown). PLC<sub>δ1</sub>PH-GFP, on the other hand, accumulated strongly at

the plasma membrane and had a low and homogenous distribution in the cytosol (Figure 1A, 1B, left panel), consistent with the hypothesis that the large pool of PtdIns(4,5)P<sub>2</sub> exists in the plasma membrane<sup>[13]</sup>. Next we examined the effects of ACh and BK, acting through their respective G protein-linked receptors, and subsequent activation of phospholipase C<sub>β</sub> and hydrolysis of PtdIns(4,5)P<sub>2</sub>, on the fluorescence distribution of the GFP and PLC<sub>δ</sub><sub>1</sub>PH-GFP. COS-7 cells were transfected with the GFP or PLC<sub>1</sub>PH-GFP together with the cDNA encoding the M<sub>1</sub>R or BK<sub>2</sub>R. After stimulation with either ACh (5 μmol/L) or BK (0.1 μmol/L), there was a decrease of PLC<sub>δ</sub><sub>1</sub>PH-GFP fluorescence in the plasma membrane and a concomitant increase in cytosolic fluorescence (Figure 1A, 1B, Table 1). The kinetics of ACh- or BK-induced PLC<sub>δ</sub><sub>1</sub>PH-GFP fluorescence translocation were characterized by a rapid onset, with translocation peaking at approximately 30–60 s and returning to baseline approximately 5–8 min after washout (Figure 1C). No significant change in fluorescence were seen in cells transfected with GFP only (data not

**Table 1.** Effects of ACh and its solvent on change in relative fluorescence ratios of PLC<sub>δ</sub><sub>1</sub>PH-GFP. COS-7 cells were transfected with a PLC<sub>δ</sub><sub>1</sub>PH-GFP fusion construct and M<sub>1</sub>R. *F<sub>m</sub>*, average plasma membrane fluorescence intensity; *F<sub>c</sub>*, average cytosolic fluorescence intensity, normalized to 1 at time 0 s for each cell. The solvent of ACh was a modified Krebs-Ringer buffer solution. *n*=5. Mean±SD. <sup>c</sup>*P*<0.01 vs control. <sup>f</sup>*P*<0.01 vs ACh at the same time point.

Drug	Time/s	Relative fluorescence ratios ( <i>F<sub>m</sub></i> / <i>F<sub>c</sub></i> )
ACh	0 (Control)	1.00±0.00
	50	0.20±0.12 <sup>c</sup>
	100	0.23±0.14 <sup>c</sup>
	500	0.95±0.05
ACh solvent	0 (Control)	1.00±0.00
	50	1.00±0.09 <sup>f</sup>
	100	1.00±0.09 <sup>f</sup>
	500	1.00±0.09



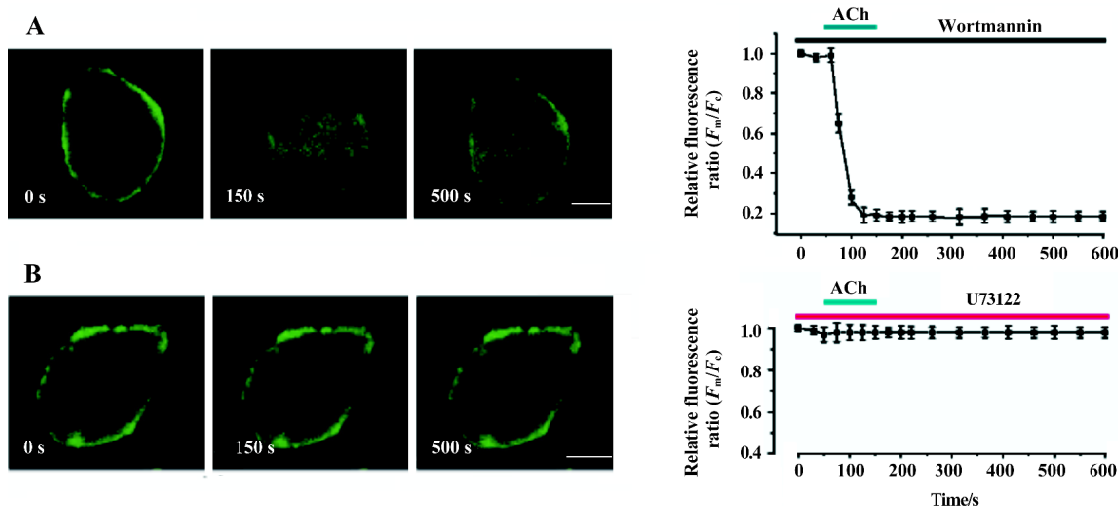
**Figure 1.** Activation of M<sub>1</sub>R induces reversible translocation of PLC<sub>δ</sub><sub>1</sub>PH-GFP from the membrane to the cytosol. COS-7 cells were transfected with the PLC<sub>δ</sub><sub>1</sub>PH-GFP fusion construct and M<sub>1</sub>R. (A) The fluorescence intensity changes across the white lines from a representative cell. (B) Plotted as line intensity histograms. (C) Shown in upper panels are time courses of the relative fluorescence intensities in the membrane (*F<sub>m</sub>*) and cytosol (*F<sub>c</sub>*) regions. Shown in lower panels are time courses of the relative fluorescence ratios (*F<sub>m</sub>*/*F<sub>c</sub>*) and normalized to 1 at time 0 s. Bar=8 μm.

shown). To exclude the effects of the laser, we used modified Krebs-Ringer buffer solution as a solvent control of ACh. As shown in Table 1, there was no change in the relative fluorescence ratios ( $F_m/F_c$ ) in the solvent control group during perfusion. To examine whether ACh- or BK-induced translocation of PLC $_{\delta_1}$ PH-GFP is due to the hydrolysis of PtdIns(4,5)P $_2$ , we utilized 2 different PtdIns(4,5)P $_2$  resynthesis and hydrolysis blockers: wortmannin and U73122. Wortmannin is known to be able to block the PtdIns 3-kinase at low concentrations and block the PtdIns 4-kinase, so therefore block the formation of PtdIns(4,5)P $_2$  from phosphatidylinositol (PI), at high concentrations<sup>[20]</sup>. As shown in Figure 2, in COS-7 cells expressing PLC $_{\delta_1}$ PH-GFP and the M $_1$ R, after the cell was pre-incubated with wortmannin (at 10  $\mu$ mol/L, a concentration known to block PtdIns 4-kinase) for 20 min, ACh induced a similar translocation of fluorescence from the plasma membrane to the cytosol, which lasted for more than 10 min after washout of ACh (Figure 2A). When the cells were pre-incubated with U73122 (10  $\mu$ mol/L, a relatively specific PLC inhibitor, for 5 min, ACh failed to induce transient translocation of the fluorescence signal (Figure 2B). These data strongly suggest that PLC $_{\delta_1}$ PH-GFP translocation induced by membrane receptor activation is indeed due to PtdIns(4,5)P $_2$  hydrolysis.

**Expression of PLC $_{\delta_1}$ PH-GFP inhibited the effects of neomycin on PtdIns(4,5)P $_2$  hydrolysis** Neomycin binds PtdIns(4,5)P $_2$  with high affinity and has often been used as an inhibitor of PLC. The blocking effect of neomycin on PLC is believed to be indirect, the result of neomycin binding to

PtdIns(4,5)P $_2$ , the substrate of PLC<sup>[15]</sup>. As we shown earlier, PLC $_{\delta_1}$ PH bound to PtdIns(4,5)P $_2$  but did not block receptor-mediated PLC activation, or PtdIns(4,5)P $_2$  hydrolysis. Because both PLC $_{\delta_1}$ PH and neomycin bind PtdIns(4,5)P $_2$  in a similar way (electrostatic interaction, see Introduction), we thought this difference between PLC $_{\delta_1}$ PH and neomycin was interesting, and worthy of further investigation. To determine whether the binding of PLC $_{\delta_1}$ PH-GFP to PtdIns(4,5)P $_2$  can disrupt the effects of neomycin on PtdIns(4,5)P $_2$  hydrolysis, COS-7 cells expressing PLC $_{\delta_1}$ PH-GFP and M $_1$ R were stimulated with ACh in the absence or presence of neomycin. Preincubation of the cells with neomycin (5 mmol/L) for 40 min failed to prevent the release of the fluorescence signal from the membrane to the cytosol upon the application of ACh (Figure 3). Thus in the presence of PLC $_{\delta_1}$ PH, neomycin could not block hydrolysis of PtdIns(4,5)P $_2$  induced by PLC.

**Effects of neomycin on PLC activation in the absence of PLC $_{\delta_1}$ PH-GFP** To further confirm that binding of PLC $_{\delta_1}$ PH-GFP to PtdIns(4,5)P $_2$  excludes the binding of neomycin to PtdIns(4,5)P $_2$ , thus blocking neomycin's inhibitory effects on PLC, we used  $[Ca^{2+}]_i$  as an indicator to reveal the effects of neomycin on PLC in the absence of PLC $_{\delta_1}$ PH-GFP. One of the downstream products of PtdIns(4,5)P $_2$  hydrolyzed by PLC is IP $_3$ , which acts to release intracellular Ca $^{2+}$ <sup>[1,21]</sup>. Thus  $[Ca^{2+}]_i$  would serve as a good indicator of PLC activation upon membrane receptor (M $_1$ R) stimulation. ACh induced a significant increase in  $[Ca^{2+}]_i$  in COS-7 cells expressing the M $_1$ R alone and pretreated with modified Krebs-Ringer buffer

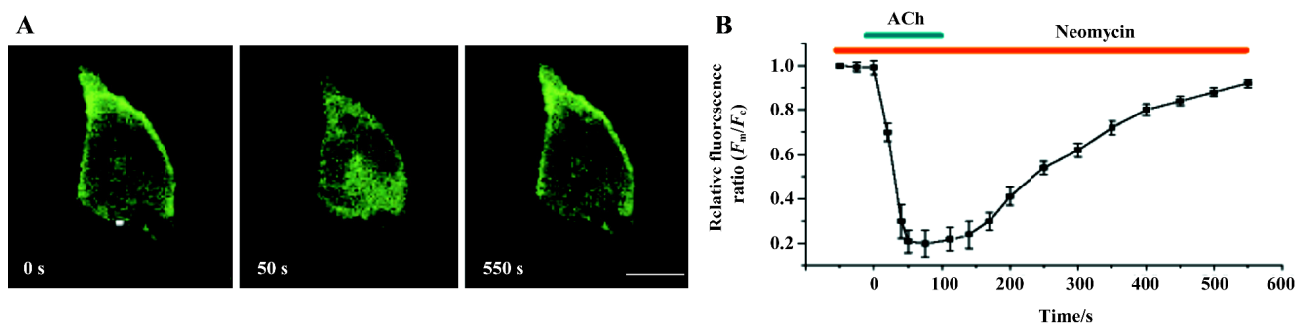


**Figure 2.** Effects of wortmannin and U73122 on translocation of PLC $_{\delta_1}$ PH-GFP. (A) Cells were preincubated with wortmannin (10  $\mu$ mol/L) for 20 min. ACh (5  $\mu$ mol/L) was applied for the indicated period of time. Shown in upper panels are confocal images from a representative cell taken at selected times after the application of ACh. The membrane:cytosol fluorescence ratios were determined (lower panels) as described earlier.  $n=5$ . Mean $\pm$ SD. Bar=8  $\mu$ m. (B) Similar experiments were performed as shown in part A, except cells were incubated with 10  $\mu$ mol/L U73122 for 5 min before experiments were carried out.

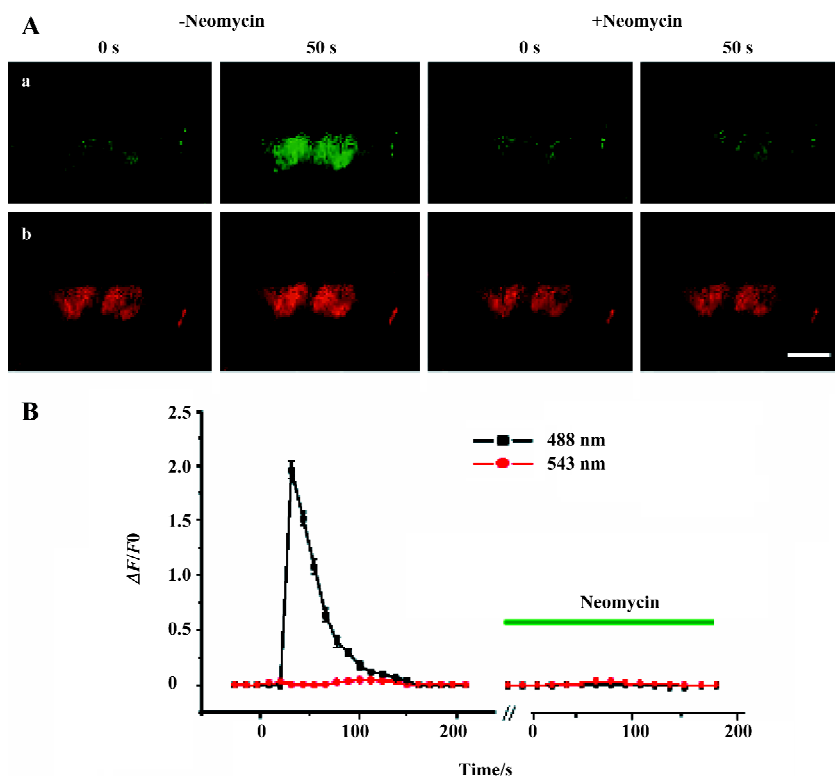
solution for 40 min (Figure 4A, 4B); pDsRed was co-transfected with M<sub>1</sub>R as a transfection tag (Figure 4A). However, when cells were pretreated with 5 mmol/L neomycin (40 min), no change was seen upon application of ACh (Figure 4A, 4B). Similar results were seen with BK as activator of PLC in cells expressing B<sub>2</sub>R (data not shown). Thus, in the absence of PLC<sub>δ1</sub>PH, neomycin was able to block activation of PLC.

To further confirm these findings, we next examined whether neomycin could also exert its inhibitory effects on [Ca<sup>2+</sup>]<sub>i</sub> in the presence of PLC<sub>δ1</sub>PH-GFP. In this section of the study, we imaged the whole-cell fluorescence intensity changes. GFP and Fluo 3-AM were excited and imaged at

the same wavelength. However, as shown in Figure 1C, the total GFP signal from one cell did not change during translocation, thus we were able to see an additional fluorescence signal from Fluo 3-AM (Ca<sup>2+</sup>) when Ca<sup>2+</sup> was released from the store by IP<sub>3</sub>. Figure 5 shows COS-7 cells transfected with PLC<sub>δ1</sub>PH-GFP and the M<sub>1</sub>R. Three types of cells, presumably representing different transfection results, can be identified. Cells designated a and b (Figure 5A) represent those cells that had been transfected with both PLC<sub>δ1</sub>PH-GFP and the M<sub>1</sub>R, giving a clear and dominant localization of the GFP signal on the cell membrane (Figure 5A), which translocated into the cytosol upon application of ACh (Figure



**Figure 3.** Neomycin failed to block PLC activation in the cells expressing PLC<sub>δ1</sub>PH-GFP. Cells were preincubated with neomycin (5 mmol/L) for 40 min before the addition of ACh (5 μmol/L) for the indicated time. (A) Confocal images from a representative cell taken at selected times after the application of ACh. (B) The membrane:cytosol fluorescence ratios were determined as described earlier. *n*=5. Mean±SD. Bar=8 μm.



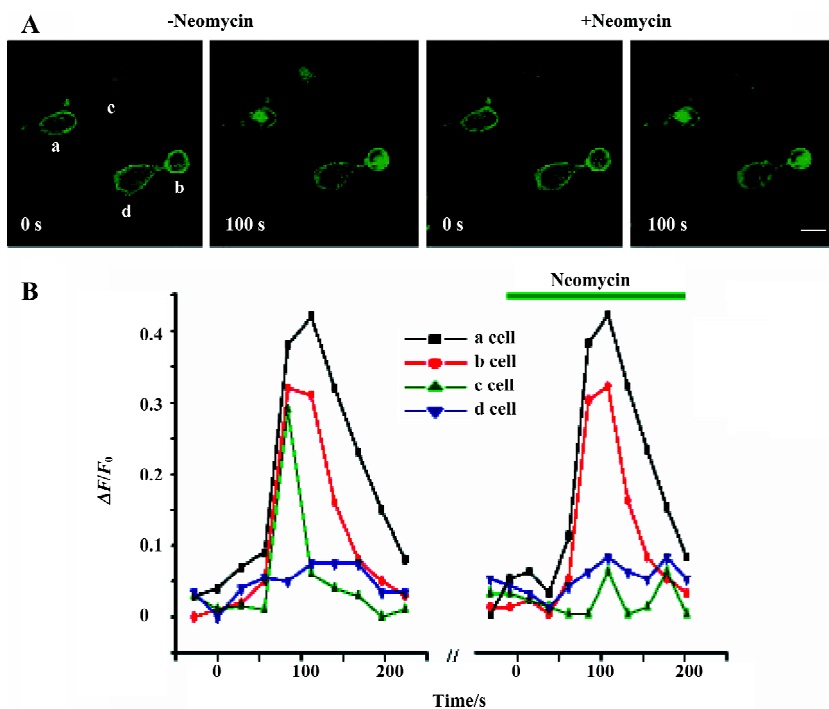
**Figure 4.** Neomycin blocked [Ca<sup>2+</sup>]<sub>i</sub> increases upon M<sub>1</sub>R activation in the cells not expressing PLC<sub>δ1</sub>PH-GFP. [Ca<sup>2+</sup>]<sub>i</sub> responses to ACh application were analyzed by confocal Ca<sup>2+</sup> imaging using Fluo-3-AM and as indicated by ΔF/F<sub>0</sub>, where ΔF and F<sub>0</sub> are the change in fluorescence intensity before and after treatment (ACh), and the initial fluorescence intensity, respectively. (A) Confocal images of a representative cell taken at selected times during the experiment. pDsRed was co-transfected with M<sub>1</sub>R as a tag of transfection (Ab). The numbers indicate the elapsed time in seconds after ACh application. (B) ΔF/F<sub>0</sub> was plotted against time; a and b refer to the same cell measured at different wavelengths. *n*=5. Mean±SD. Bar=8 μm.

5A); cell c represents cells that had only been transfected with  $M_1R$ , with no visible localization of the  $PLC_{\delta_1}PH$ -GFP signal on the cell membrane, and a clear rising in  $[Ca^{2+}]_i$  signal seen upon application of ACh; cell d represents cells that had been transfected with  $PLC_{\delta_1}PH$ -GFP but not the  $M_1R$ , so that the clear  $PLC_{\delta_1}PH$ -GFP signal was not released from the membrane, and neither could an increase in  $[Ca^{2+}]_i$  signal be seen upon application of ACh (Figure 5A). When these cells were pretreated with neomycin, only the response of cell c to ACh was blocked, whereas the responses of cells a and b were unaffected. These results strongly suggest that neomycin blocks PLC activation only in the absence of  $PLC_{\delta_1}PH$ -GFP.

## Discussion

The main finding of the present study was that in the cells expressing  $PLC_{\delta_1}PH$ -GFP, neomycin could not exhibit its inhibitory effects on  $PtdIns(4,5)P_2$  hydrolysis by PLC. There is increasing interest in understanding the actions of inositol phospholipids, especially  $PtdIns(4,5)P_2$ , in living cells<sup>[21]</sup>.  $PtdIns(4,5)P_2$  participates in many cellular functions, including exocytosis, cytoskeletal function and membrane transporter and ion channel functions<sup>[4]</sup>. Many molecules have been found to be able to bind to phospholipids, and more specifically to  $PtdIns(4,5)P_2$ , which forms the basis of modulation by this lipid<sup>[22]</sup>. The PH domain of  $PLC_{\delta_1}$  is one of these molecules that are believed to selectively bind to

$PtdIns(4,5)P_2$ <sup>[12]</sup>. Recently, a fusion construct of  $PLC_{\delta_1}PH$  with enhanced green fluorescent protein ( $PLC_{\delta_1}PH$ -GFP) was developed as a probe to visualize  $PtdIns(4,5)P_2$  in single cells. This novel methodology allowed imaging and analysis of spatiotemporal changes in  $PtdIns(4,5)P_2$  in single living cells, and has been used increasingly in efforts to understand the role  $PtdIns(4,5)P_2$  plays in cell signaling, and protein function regulation<sup>[13]</sup>. The central idea behind this methodology is that the GFP signal that has been linked to  $PLC_{\delta_1}PH$  will faithfully follow the dynamic changes of  $PtdIns(4,5)P_2$  during its metabolism, including during hydrolysis by PLC. However, because  $PLC_{\delta_1}PH$  also binds to  $IP_3$ , a downstream product of  $PtdIns(4,5)P_2$  hydrolysis, with higher affinity, some have proposed that rather than being a faithful  $PtdIns(4,5)P_2$  follower,  $PLC_{\delta_1}PH$ -GFP molecules during  $PtdIns(4,5)P_2$  hydrolysis are more likely to bind to newly produced  $IP_3$ <sup>[18]</sup>. But van der Wal *et al* showed that physiological increases in  $IP_3$  (10–100  $\mu\text{mol/L}$ ) on activation of PLC could not be solely responsible for the translocation of  $PLC_{\delta_1}PH$ -GFP<sup>[23]</sup>. Our data presented in Figure 2 are in agreement with the results of van der Wal *et al*. In the cells expressing  $PLC_{\delta_1}PH$ -GFP as well as the  $BK_2$  or  $M_1$  receptors, BK or ACh induced the reversible translocation of  $PLC_{\delta_1}PH$ -GFP from the plasma membrane to the cytosol. Thus, although it bound to  $PtdIns(4,5)P_2$ ,  $PLC_{\delta_1}PH$ -GFP did not interfere with cleavage of  $PtdIns(4,5)P_2$  by PLC. On the other hand, neomycin, a commonly used PLC blocker, is believed to block PLC cleavage of  $PtdIns(4,5)P_2$  by preventing  $PtdIns(4,5)P_2$  from



**Figure 5.** Presence or absence of  $PLC_{\delta_1}PH$ -GFP determines the effect of neomycin on PLC activation.  $[Ca^{2+}]_i$  responses to ACh application were analyzed as described in Figure 4 legend. (A) Confocal image from a representative cell taken at selected times in seconds after application of ACh. (B)  $\Delta F/F_0$  was plotted against time. Bar=8  $\mu\text{m}$ .

accessing PLC<sup>[17]</sup>. It is interesting to note that whereas a smaller molecule such as neomycin would mask PtdIns(4,5)P<sub>2</sub> from PLC cleavage, a much bigger molecule such as PLC<sub>δ1</sub>PH-GFP would allow the cleavage to happen. It is also interesting to consider that the expression of PLC<sub>δ1</sub>PH-GFP blocked the action of neomycin (Figure 3), suggesting that PLC<sub>δ1</sub>PH-GFP and neomycin bind to the same sites on PtdIns(4,5)P<sub>2</sub>. Previous studies have demonstrated that both PLC<sub>δ1</sub>PH-GFP and neomycin interact with PtdIns(4,5)P<sub>2</sub> in an electrostatic way<sup>[12,14]</sup>. Thus the charged inositide head group of PtdIns(4,5)P<sub>2</sub> is the binding site for both PLC<sub>δ1</sub>PH-GFP and neomycin<sup>[24]</sup>, yet binding of PLC<sub>δ1</sub>PH-GFP or neomycin to PtdIns(4,5)P<sub>2</sub> has very different consequences for PLC hydrolysis of PtdIns(4,5)P<sub>2</sub>. Although it is less likely, it needs to be noted that the GFP, rather than PLC<sub>δ1</sub>PH, may block the binding of neomycin to PtdIns(4,5)P<sub>2</sub> through a spatial blocking effect. For many cellular proteins that have been known to interact with, and whose functions are regulated by, PtdIns(4,5)P<sub>2</sub>, the molecular basis for the interaction remains to be elucidated. Less clear is the mechanism for PtdIns(4,5)P<sub>2</sub> hydrolysis by PLC. The present study provides interesting and stimulating information for further understanding protein-PtdIns(4,5)P<sub>2</sub> interactions and PtdIns(4,5)P<sub>2</sub> hydrolysis by PLC. We are currently investigating the mechanism underlying the different consequences of PtdIns(4,5)P<sub>2</sub> binding to PLC<sub>δ1</sub>PH-GFP or neomycin with respect to its hydrolysis by PLC.

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