Acta Pharmacologica Sinica 2005 Dec; 26 (12): 1485–1491

Blackwell Publishing

Full-length article

Binding of PLCδ_1PH-GFP to PtdIns(4,5)P_2 prevents inhibition of phospholipase C-mediated hydrolysis of PtdIns(4,5)P_2 by neomycin[1]

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Key words

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

[1] Project supported by the National Natural Science Foundation of China (No 30270361), Ministry of Science and Technology of China (No 2003CCA00300), HL ZHANG is a beneficiary of the National Science Fund for Distinguished Young Scholars of China (No 30325038).

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Received 2005-02-22
Accepted 2005-07-06

doi: 10.1111/j.1745-7254.2005.00223.x

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^{2+} release from intracellular Ca^{2+} stores, and the protein kinase C activator, diacylglycerol[1,2]. 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[3]. PtdIns(4,5)P_2 itself is a regulator of a great variety of target molecules, including ion channels[4,5] and several proteins that regulate actin polymerization and the cytoskeleton[6], providing a link between the plasma membrane and the cortical cytoskeleton[7]. 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[8], clathrin-mediated endocytosis[9], and membrane recovery by endocytosis during neurotransmitter release[10]. 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[11]. The PH domain of phospholipase $C_{\delta 1}$ ($PLC_{\delta 1}PH$) binds with high affinity and selectivity to PtdIns(4,5)P_2[12]. 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_2 is resynthesized, fluorescence returns to the membrane[13].

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_2[14]. Later studies also showed that neomycin bound to and neutralized the negative charge of PtdIns(4,5)P_2[15].

Phospholipase C (PLC)-induced PtdIns(4,5)P_2 hydrolysis is an important cell signaling mechanism. Many membrane receptors couple to PLC, and thus regulate PtdIns(4,5)P_2 turnover and subsequent downstream cell signaling[16]. 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_2. Neomycin has long been used as a blocker of PLC, although it actually binds to PtdIns(4,5)P_2 and presumably prevents PtdIns(4,5)P_2 from hydrolysis by PLC[17]. Previous studies have demonstrated that both $PLC_{\delta 1}$PH and neomycin bind PtdIns(4,5)P_2 through an electrostatic interaction[12,14]. This similar nature of interaction would indicate a similar consequence for PtdIns(4,5)P_2 hydrolysis by PLC. However, in the present study, we demonstrate that although both PLCδ1PH and neomycin bind to PtdIns(4,5)P_2, only neomycin blocks PtdIns(4,5)P_2 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_2SO), with a final concentration of Me_2SO 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_1) receptor (M_1R) and bradykinin 2 receptor (BK_2R), pEGFP-N1(GFP) and the $PLC_{\delta 1}$PH construct with GFP ($PLC_{\delta 1}$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_2 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 $MgSO_4$, 1.2 $CaCl_2$, 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 $PLC_{\delta 1}$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^{2+} ($[Ca^{2+}]_i$) 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. $[Ca^{2+}]_i$ changes were represented by relative fluorescence intensity calculated by using the equation $\Delta F/F_0$, where ΔF and F_0 are the change in fluorescence intensity before and after treatment, and the initial fluorescence intensity, respectively[18,19].

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_1R and BK_2R induced PtdIns(4,5)P_2 hydrolysis and a reversible translocation of $PLC_{\delta 1}$PH-GFP Both M_1R, and $PLC_{\delta 1}$PH-GFP or both BK_2 and $PLC_{\delta 1}$PH-GFP were expressed in COS-7 cells. To follow the localization of $PLC_{\delta 1}$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). $PLC_{\delta 1}$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_2 exists in the plasma membrane[13]. Next we examined the effects of ACh and BK, acting through their respective G protein-linked receptors, and subsequent activation of phospholipase C_β and hydrolysis of PtdIns(4,5)P_2, on the fluorescence distribution of the GFP and $PLC_{\delta1}$PH-GFP. COS-7 cells were transfected with the GFP or PLC_1PH-GFP together with the cDNA encoding the M_1R or BK_2R. After stimulation with either ACh (5 μmol/L) or BK (0.1 μmol/L), there was a decrease of $PLC_{\delta1}$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 $PLC_{\delta1}$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 $PLC_{\delta1}$PH-GFP. COS-7 cells were transfected with a $PLC\delta_1$PH-GFP fusion construct and M_1R. F_m, average plasma membrane fluorescence intensity; F_c, 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. $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* ACh at the same time point.

Drug	Time/s	Relative fluorescence ratios (F_m/F_c)
ACh	0 (Control)	1.00±0.00
	50	0.20±0.12^c
	100	0.23±0.14^c
	500	0.95±0.05
ACh solvent	0 (Control)	1.00±0.00
	50	1.00±0.09^f
	100	1.00±0.09^f
	500	1.00±0.09

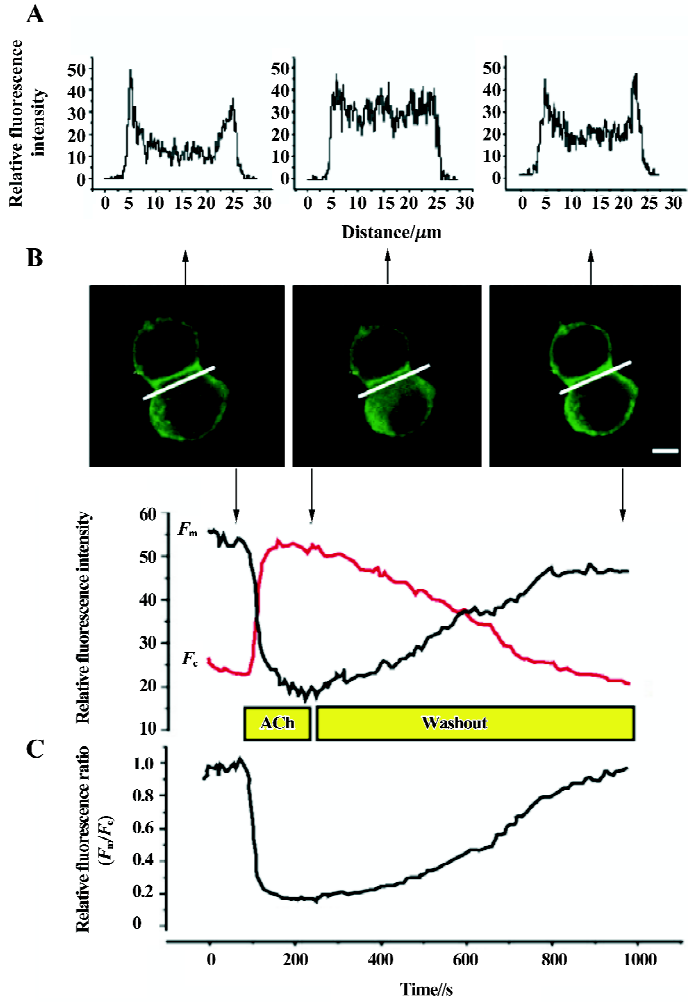

Figure 1. Activation of M_1R induces reversible translocation of $PLC_{\delta1}$PH-GFP from the membrane to the cytosol. COS-7 cells were transfected with the $PLC_{\delta1}$PH-GFP fusion construct and M_1R. (A) The fluorescence intensity changes across the white lines from a representative cell. (B) Plotted as line intensity histograms. (C) Shown in upper panels are time courses of the relative fluorescence intensities in the membrane (F_m) and cytosol (F_c) regions. Shown in lower panels are time courses of the relative fluorescence ratios (F_m/F_c) 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[20]. As shown in Figure 2, in COS-7 cells expressing $PLC_{\delta 1}PH$-GFP and the M_1R, after the cell was pre-incubated with wortmannin (at 10 μmol/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 μmol/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[15]. 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_1R 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+}[1,2]. Thus $[Ca^{2+}]_i$ would serve as a good indicator of PLC activation upon membrane receptor (M_1R) stimulation. ACh induced a significant increase in $[Ca^{2+}]_i$ in COS-7 cells expressing the M_1R alone and pretreated with modified Krebs-Ringer buffer

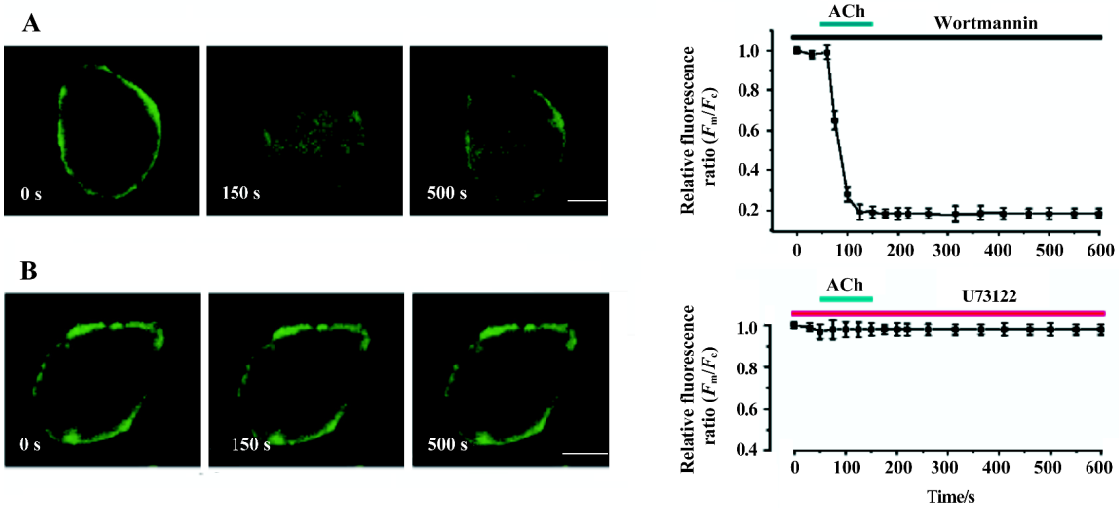

Figure 2. Effects of wortmannin and U73122 on translocation of $PLC_{\delta 1}PH$-GFP. (A) Cells were preincubated with wortmannin (10 μmol/L) for 20 min. ACh (5 μmol/L) was applied for the indicated period of time. Shown in upper panels are confocal images from a representative cell taken at selected times after the application of ACh. The membrane:cytosol fluorescence ratios were determined (lower panels) as described earlier. n=5. Mean±SD. Bar=8 μm. (B) Similar experiments were performed as shown in part A, except cells were incubated with 10 μmol/L U73122 for 5 min before experiments were carried out.

solution for 40 min (Figure 4A, 4B); pDsRed was co-transfected with M_1R 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_2R (data not shown). Thus, in the absence of $PLC_{\delta 1}PH$, 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 $[Ca^{2+}]_i$ in the presence of $PLC_{\delta 1}PH$-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^{2+}) when Ca^{2+} was released from the store by IP_3. Figure 5 shows COS-7 cells transfected with $PLC_{\delta 1}PH$-GFP and the M_1R. 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 $PLC_{\delta 1}PH$-GFP and the M_1R, 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

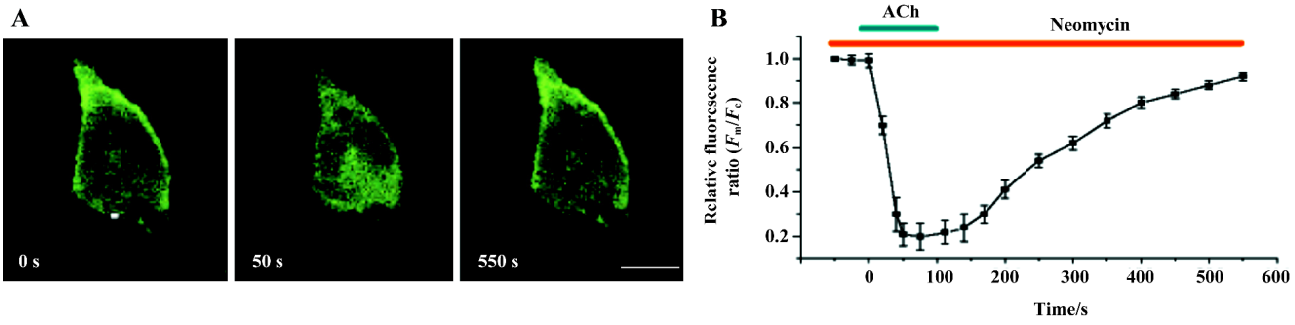

Figure 3. Neomycin failed to block PLC activation in the cells expressing $PLC_{\delta 1}PH$-GFP. Cells were preincubated with neomycin (5 mmol/L) for 40 min before the addition of ACh (5 μmol/L) for the indicated time. (A) Confocal images from a representative cell taken at selected times after the application of ACh. (B)The membrane:cytosol fluorescence ratios were determined as described earlier. n=5. Mean±SD. Bar=8 μm.

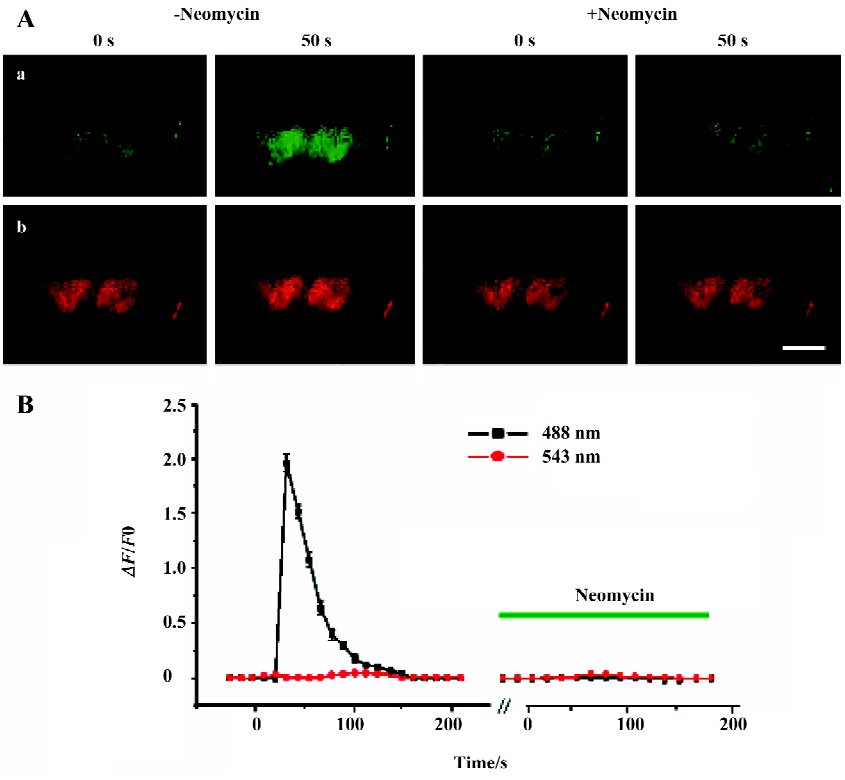

Figure 4. Neomycin blocked $[Ca^{2+}]_i$ increases upon M_1R activation in the cells not expressing $PLC\delta_1PH$-GFP. $[Ca^{2+}]_i$ responses to ACh application were analyzed by confocal Ca^{2+} imaging using Fluo-3-AM and as indicated by $\Delta F/F_0$, where ΔF and F_0 are the change in fluorescence 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_1R as a tag of transfection (Ab). The numbers indicate the elapsed time in seconds after ACh application. (B) $\Delta F/F_0$ 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 $PLC_{\delta1}PH$-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 $PLC_{\delta1}PH$-GFP but not the M_1R, so that the clear $PLC_{\delta1}PH$-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 $PLC_{\delta1}PH$-GFP.

Discussion

The main finding of the present study was that in the cells expressing $PLC_{\delta1}PH$-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[21]. PtdIns(4,5)P_2 participates in many cellular functions, including exocytosis, cytoskeletal function and membrane transporter and ion channel functions[4]. 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[22]. The PH domain of $PLC_{\delta1}$ is one of these molecules that are believed to selectively bind to PtdIns(4,5)P_2[12]. Recently, a fusion construct of $PLC_{\delta1}PH$ with enhanced green fluorescent protein ($PLC_{\delta1}PH$-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[13]. The central idea behind this methodology is that the GFP signal that has been linked to $PLC_{\delta1}PH$ will faithfully follow the dynamic changes of PtdIns(4,5)P_2 during its metabolism, including during hydrolysis by PLC. However, because $PLC_{\delta1}PH$ 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, $PLC_{\delta1}PH$-GFP molecules during PtdIns(4,5)P_2 hydrolysis are more likely to bind to newly produced IP_3[18]. But van der Wal *et al* showed that physiological increases in IP_3 (10–100 μmol/L) on activation of PLC could not be solely responsible for the translocation of $PLC_{\delta1}PH$-GFP[23]. Our data presented in Figure 2 are in agreement with the results of van der Wal *et al*. In the cells expressing $PLC_{\delta1}PH$-GFP as well as the BK_2 or M_1 receptors, BK or ACh induced the reversible translocation of $PLC_{\delta1}PH$-GFP from the plasma membrane to the cytosol. Thus, although it bound to PtdIns(4,5)P_2, $PLC_{\delta1}PH$-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

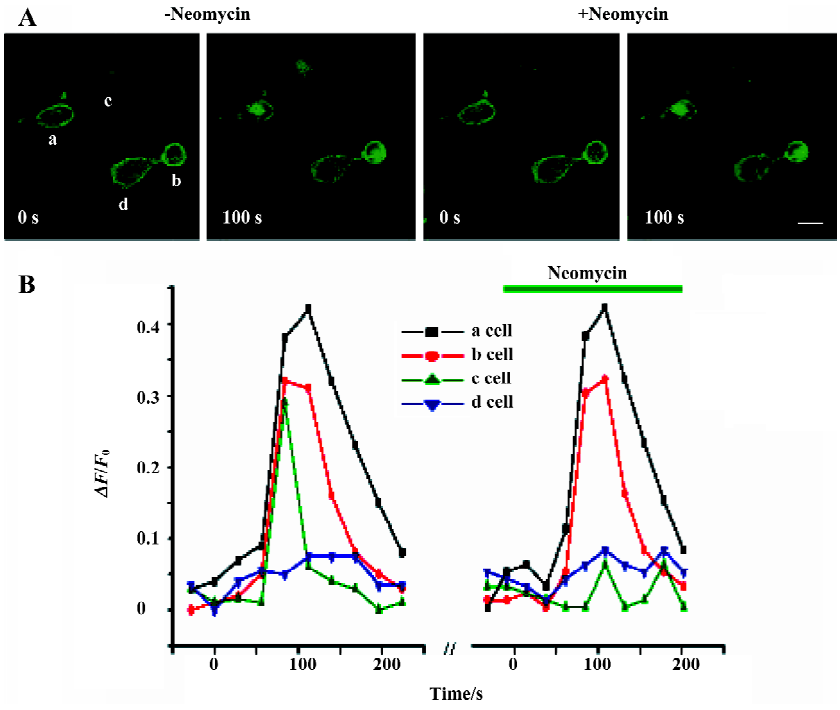

Figure 5. Presence or absence of $PLC_{\delta1}PH$-GFP determines the effect of neomycin on PLC activation. $[Ca^{2+}]_i$ responses to ACh application were analyzed as described in Figure 4 legend. (A) Confocal image from a representative cell taken at selected times in seconds after application of ACh. (B) $\Delta F/F_0$ was plotted against time. Bar=8 μm.

accessing PLC[17]. It is interesting to note that whereas a smaller molecule such as neomycin would mask PtdIns(4,5)P_2 from PLC cleavage, a much bigger molecule such as $PLC_{\delta 1}$PH-GFP would allow the cleavage to happen. It is also interesting to consider that the expression of $PLC_{\delta 1}$PH-GFP blocked the action of neomycin (Figure 3), suggesting that $PLC_{\delta 1}$PH-GFP and neomycin bind to the same sites on PtdIns(4,5)P_2. Previous studies have demonstrated that both $PLC_{\delta 1}$PH-GFP and neomycin interact with PtdIns(4,5)P_2 in an electrostatic way[12,14]. Thus the charged inositide head group of PtdIns(4,5)P_2 is the binding site for both $PLC_{\delta 1}$PH-GFP and neomycin[24], yet binding of $PLC_{\delta 1}$PH-GFP or neomycin to PtdIns(4,5)P_2 has very different consequences for PLC hydrolysis of PtdIns(4,5)P_2. Although it is less likely, it needs to be noted that the GFP, rather than $PLC_{\delta 1}$PH, may block the binding of neomycin to PtdIns(4,5)P_2 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_2, the molecular basis for the interaction remains to be elucidated. Less clear is the mechanism for PtdIns(4,5)P_2 hydrolysis by PLC. The present study provides interesting and stimulating information for further understanding protein-PtdIns(4,5)P_2 interactions and PtdIns(4,5)P_2 hydrolysis by PLC. We are currently investigating the mechanism underlying the different consequences of PtdIns(4,5)P_2 binding to $PLC_{\delta 1}$PH-GFP or neomycin with respect to its hydrolysis by PLC.

Acknowledgements

We thank Diomedes E LOGOTHETIS (Mount Sinai School of Medicine, New York University, NY, USA), for providing us with plasmids of the M_1R, BK_2R and $PLC_{\delta 1}$PH-GFP, and members of the Logothetis laboratory for helpful discussions on this work.

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