

Full Paper

Inhibitory Effect of the Phosphoinositide 3-Kinase Inhibitor LY294002 on Muscarinic Acetylcholine Receptor-Induced Calcium Entry in PC12h Cells

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Abstract. Phosphoinositide-3 kinase (PI3K) and phospholipase C (PLC) utilize the same phosphoinositides as substrates to produce different signaling molecules. These enzymes are activated by a similar set of cell signaling mechanisms, i.e., tyrosine kinases and G proteins, and affect common cell functions, including proliferation, motility, and intracellular trafficking. Despite these similarities, the interplay between these enzymes is not well understood. To address this issue, the effects of the PI3K inhibitor LY294002 on carbachol-induced calcium increase in PC12h cells were examined. As carbachol stimulates both Gq- and Gi-coupled muscarinic acetylcholine receptors (mAChRs), PI3K and PLC are activated simultaneously in this protocol. LY294002 was found to reduce the carbachol-induced calcium increase, and the reduction was attributed to suppression of calcium entry. As LY294002 did not affect either carbachol-induced calcium release or calcium entry induced by calcium store depletion, this agent was found to suppress calcium entry directly activated by mAChRs. Although PI3K was supposed to compete for substrates with PLC, the PI3K inhibitor did not enhance PLC-dependent cellular responses. As LY294002 was still effective by treating cells after carbachol stimulation, it is likely that this agent blocks the calcium entry channels directly.

Keywords: calcium entry, phosphoinositide-3 kinase, muscarinic acetylcholine receptor, PC12, phospholipase C

Introduction

Phosphoinositide 3-kinase (PI3K) phosphorylates the 3'-OH position of the inositol ring of inositol phospholipids, and produces phosphatidylinositol (3) phosphate (PtdIns(3)P), PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃ (1). Diverse signaling proteins containing pleckstrin homology, FYVE, Phox, and other lipid-binding domains bind to the PI3K products and are recruited to the signaling protein complex (2 – 5). PI3K is activated by tyrosine kinase or heterotrimeric G protein signaling and plays key roles in a wide range of cellular functions, including cell growth, proliferation, motility differentiation, survival, and intracellular

trafficking (2, 6 – 8). Two structurally unrelated PI3K inhibitors, wortmannin and LY294002 (9, 10), have been used in pharmacological analyses and the results have revealed linkages between PI3K and human diseases, i.e., allergy inflammation, heart disease, and cancer (11 – 14).

Phosphoinositide hydrolysis by phospholipase C (PLC), which produces the intracellular calcium mobilizing second messenger inositol 1,4,5-trisphosphate (IP₃) and the protein kinase C activating second messenger diacylglycerol, is another important cell signaling mechanism by phosphoinositide metabolism. PLC and PI3K share substrates and activation pathways, i.e., tyrosine kinases and G proteins, and are involved in common cell functions, i.e., proliferation, motility, and secretion. Therefore, the crosstalk between these enzymes is considered to enable fine control of cellular activities. Although it is assumed that these two enzymes

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are activated simultaneously and interfere with each other by competing for substrates, tyrosine kinase-dependent activation of both enzymes in immune cells has been shown to be essential for calcium mobilization (15). With regard to the G protein-dependent activation pathway, there have been few studies on the crosstalk between PLC and PI3K, and the entire picture of phosphoinositide metabolism-mediated cell function remains to be elucidated.

In the present study, the effects of the PI3K inhibitor LY294002 were examined on increases in calcium in PC12h cells induced by the muscarinic acetylcholine receptor (mAChR) agonist carbachol. Previously, we showed that carbachol increased intracellular IP₃ and calcium in this cell line (16). As PC12-derived cell lines express both G_q- and G_i-coupled mAChRs (17, 18), which activate PI3K when expressed (19, 20), carbachol presumably activates PLC and PI3K simultaneously. Therefore, the carbachol-induced calcium increase pattern reflects crosstalk between PI3K and PLC, and the effects of LY294002 on this response will reveal the contribution of PI3K to PLC-mediated cell functions.

Materials and Methods

Cell culture and intracellular calcium measurement

PC12h cells were cultured in Dulbecco's Modified Eagle's medium High (Asahi Technoglass, Funabashi) containing 5% horse serum (Gibco-BRL, Gaithersburg, MD, USA) and 5% semi-fetal calf serum (Mitsubishi Kagaku, Tokyo) and then seeded in 96-well plates (Costar 3610; Costar, Corning, NY, USA) coated with polyethyleneimine (1 mg/ml), at 2×10^4 cells/well. Intracellular calcium measurements were performed 48–96 h after seeding. The extracellular solution for physiological experiments was basal salt solution (BSS; 130 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES, pH 7.4) or calcium-free BSS containing 0.5 mM EGTA in place of calcium. To load the calcium indicator, the cells were washed three times in BSS, incubated for 45 min at 30°C in BSS containing Fura2-AM (7.5 μM; Dojin, Kumamoto), washed three times, and then incubated at room temperature for 20 min before calcium measurements. Sulfinpyrazone (100 μM), which inhibits leakage of Fura2, and the nicotinic acetylcholine receptor antagonist hexamethonium (0.5 mM) was added to the BSS in all steps after the final wash. Intracellular calcium measurements were conducted using Act Cell Scan (Sawarabi Ltd., Tokyo) equipped with injectors and a fluorometer for microtiter plates. The calcium response in each well, which contained 200 μl extra-

cellular solution, was induced by injecting 20 μl of 10× concentrated stimulus solution, and sets of Fura2 fluorescence signals at 510 nm by exciting at 340 and 380 nm were obtained at 1-s intervals. The ratio (340/380) was calculated using the fluorescent intensity after subtracting the background, which was the fluorescence of wells without cells.

Materials

LY294002 was from Promega (Madison, WI, USA). Unless otherwise indicated, chemicals were from Sigma (St. Louis, MO, USA).

Results

Increases in intracellular calcium concentration of PC12h cells following pharmacological treatments were measured using a novel device, Act Cell Scan equipped with a fluorescence detector, and an injector for introducing liquid into 96-well microtiter plates. As shown in Fig. 1A, the EC₅₀ of the carbachol-induced calcium increase in PC12h cells was 25 ± 15 μM, which was similar to that in the previous study (21). Therefore, this high throughput screening device was shown to provide comparable results to those obtained by conventional calcium imaging. PC12 cells are known to express muscarinic- and nicotinic-acetylcholine receptors (nAChR), both of which are thought to mediate carbachol-induced increases in calcium levels. However, nAChR expression is highly dependent on nerve growth factor treatment (22), and the contribution of nAChR to the carbachol-induced calcium increase is assumed to vary between PC12-derived cell lines. To address the contribution of each receptor subtype, the effects of an nAChR antagonist hexamethonium (0.5 mM) and the mAChR antagonist atropine (2 μM) were examined. As shown in Fig. 1B (left), the carbachol-induced calcium increase was completely inhibited when both blockers were included in the extracellular solution. Although hexamethonium alone did not significantly affect the calcium increase, a small transient calcium increase remained when atropine alone was included (Fig. 1B, left). These results suggest that the atropine-insensitive fraction was the nAChR component, which was too small to be distinguished by hexamethonium treatment. In the following experiments, carbachol was used at 1 mM, which is a concentration sufficient for activation of G_i-coupled mAChRs (19), and hexamethonium was included in the extracellular solution.

The effects of the PI3K inhibitor LY294002 on the mAChR-induced calcium increase in PC12h cells were examined. The carbachol-induced calcium increase

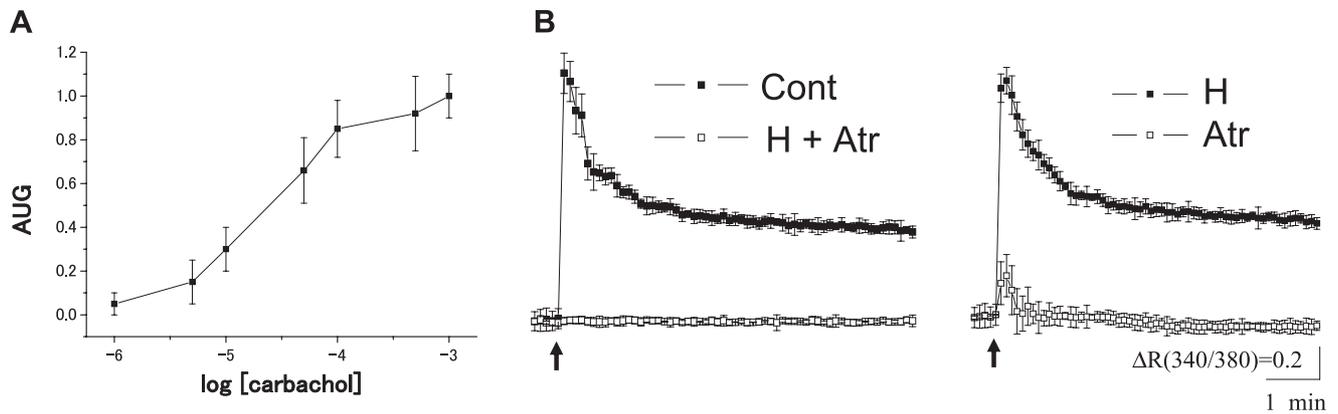


Fig. 1. Pharmacological properties of calcium increase measured using Act Cell Scan in PC12h cells following carbachol treatment. A: Carbachol-concentration-dependency. Relative area under the graph (AUG) of calcium increase during 5 min of carbachol stimulation. Means \pm S.E.M. ($n = 6$). B: Effects of acetylcholine receptor antagonists. Hexamethonium (0.5 mM, H) and atropine (2 μ M, Atr) treatment started 2 min prior to 1 mM carbachol stimulation (arrows). Traces indicate the means \pm S.E.M. of three calcium responses.

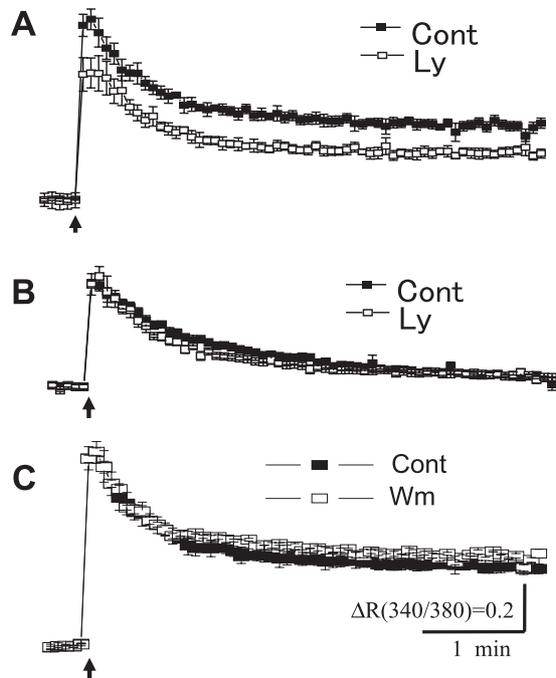


Fig. 2. Effects of PI3 kinase inhibitors on the carbachol-induced calcium increase in PC12h cells. Cells were stimulated with 1 mM carbachol (arrows). Traces indicate the means \pm S.E.M. of three calcium responses. Effects of 50 μ M LY294002 (Ly) treatment started 2 min prior to carbachol stimulation in normal BSS (A) or calcium-free BSS (B). Effects of wortmannin (100 nM, Wm) in normal BSS (C).

was partially suppressed by treatment with 50 μ M LY294002, which started 2 min prior to stimulation (Fig. 2A). In contrast, LY294002 did not affect the carbachol response in the absence of extracellular calcium (Fig. 2B). These results suggest that LY294002

affected carbachol-induced calcium entry, but not release from intracellular stores. In contrast, another IP3K inhibitor, wortmannin, at a concentration of 100 nM at which it has been reported to block PI3K activity in PC12 cells (23), did not have any effect on calcium entry, as shown in Fig. 2C, suggesting LY294002 affected calcium entry via a pathway independent of PI3K.

The effect of LY294002 was further examined using a stimulation protocol to dissect calcium entry. PC12h cells were pretreated with carbachol in calcium-free extracellular solution, and calcium entry was induced by increasing the extracellular calcium concentration. As there was no calcium entry without carbachol (data not shown) as shown previously using calcium imaging (24), the calcium entry reflected mAChR activation and/or store depletion. As shown in Fig. 3, LY294002 reduced calcium entry, and this suppressive effect was not dependent on whether LY294002 treatment was started before or after carbachol treatment. These results suggest that LY294002 is capable of blocking calcium entry channels, even after the channels had been activated. As shown in Fig. 3C, the inhibitory effect was saturated at 50–100 μ M, and IC_{50} was $10.2 \pm 5.6 \mu$ M. These observations indicated that LY294002 suppressed calcium entry at a concentration similar to that at which it inhibited PI3K (25).

mAChR-induced calcium entry in PC12 cells was shown to consist of two components: one activated by calcium store depletion following calcium release (store-operated calcium entry) and another activated by mAChR-induced diacylglycerol production (receptor-induced calcium entry) (26, 27). To address the LY294002-sensitive calcium entry, the effects of

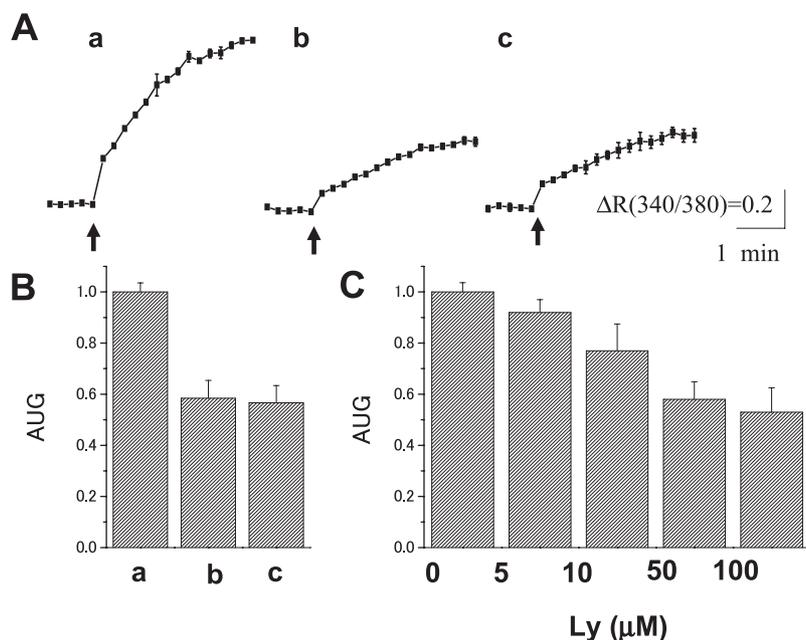


Fig. 3. Effects of the PI3 kinase inhibitor LY294002 (Ly) on carbachol-induced calcium entry in PC12h cells. A: Representative calcium response. Cells were treated with 1 mM carbachol in calcium-free BSS 3 min prior to calcium measurement. Calcium entry was induced by adding calcium (final concentration of 2 mM, indicated by the arrows) in the absence (a) or presence of Ly treatment, which started 2 min prior to carbachol treatment (b) or 1 min after starting carbachol treatment (c). Traces indicate the means \pm S.E.M. of three calcium responses. B: Relative areas under the graph (AUG) of calcium entry traces in panel A. Means \pm S.E.M. (n = 6). C: Concentration-dependent effects of Ly. Cells were treated with Ly as for panel A, and each calcium entry was presented as for panel B. Means \pm S.E.M. (n = 6).

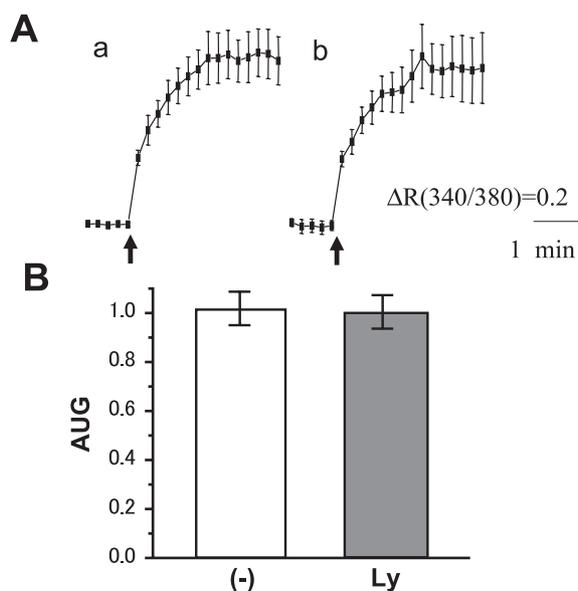


Fig. 4. Effects of the PI3 kinase inhibitor LY294002 (Ly) on intracellular store depletion-induced calcium entry in PC12h cells. A: Representative calcium response. Cells were treated with 1 μ M Tg in calcium-free BSS, 5 min prior to calcium measurement. Calcium entry was induced by adding calcium (final concentration of 2 mM, indicated by the arrows) in the absence (a) or presence of Ly treatment (b), which started 2 min prior to adding calcium. Traces indicate the means \pm S.E.M. of three calcium responses. B: Relative area under the graph (AUG) of calcium entry of panel A, a and b. Means \pm S.E.M. (n = 6).

LY294002 on the store-operated calcium entry were examined. Store-operated calcium entry was induced by depletion of intracellular stores by thapsigargin treat-

ment in calcium-free extracellular solution and subsequent increase in extracellular calcium level. LY294002 did not affect the store-operated calcium entry (Fig. 4). Therefore, this agent was suggested to suppress receptor-induced calcium entry.

Discussion

The present study examined the effects of the PI3K inhibitor LY294002 on carbachol-induced intracellular calcium increase in PC12h cells, in which Gq- and Gi-coupled mAChRs are considered to activate PLC and/or PI3K. LY294002 did not affect carbachol-induced calcium release from intracellular calcium stores, but partially suppressed carbachol-induced calcium entry. As the store-operated calcium entry was unaffected by LY294002, this agent inhibited receptor-induced calcium entry. LY294002 still suppressed calcium entry after carbachol stimulation, suggesting that this agent blocked calcium entry channels previously activated by mAChR.

LY294002 did not affect carbachol-induced calcium release, suggesting that PI3K did not influence IP3 production by PLC and subsequent calcium release. As both PLC and PI3K utilize the same substrate, PtdIns(4,5)P₂ for active products, it was hypothesized that substrate competition and interference would lead to lower levels of calcium release. The lack of enhancing effects of LY294002 on calcium release indicated that this was not the case. Activation of Gq-coupled receptors is known to induce rapid and large-scale

PtdIns(4,5)P₂ resynthesis (28). Therefore, sufficient substrate may be supplied to both enzymes after agonist stimulation. In addition, the proportion of PtdIns(3,4,5)P₃ in PC12 cells total phospholipid had been reported not to exceed 1% even after NGF treatment (23). If this is also the case for PI3K activation by carbachol, substrate depletion by PI3K was almost negligible. In the calcium release experiments (Fig. 1B), the fluorescent calcium indicator was not saturated and was still capable of measuring larger calcium increases because the amplitude was smaller than that of carbachol-induced total calcium increase (Fig. 1A). Therefore, LY294002 did not affect the quantity of calcium release, suggesting that IP₃ production by mAChRs was sufficient to release all the calcium in intracellular stores, even if PI3K interfered with PLC activity.

Carbachol had been shown to induce two independent calcium entry pathways in PC12 cells: store-operated calcium entry and receptor-induced calcium entry (26, 27). Trp6 channels have been shown to correspond to the sites of receptor-induced calcium entry (27). Therefore, LY294002 is likely to affect calcium entry via Trp6. PI3K products are poor substrates for PLC (29). Therefore, inhibition of PI3K by LY294002 is likely to supply more substrate for PLC to produce diacylglycerol, which transmits receptor signals to Trp6 and then enhances calcium entry. However, the results were the opposite of this, suggesting that the suppressive effect of LY294002 on calcium entry is likely to be attributed to pathways other than that involving PI3K. LY294002 has been reported to directly block voltage-dependent potassium and calcium channels (30, 31). As LY294002 still showed a potent inhibitory effect against calcium entry after carbachol stimulation, the results of the present study support the direct blockade of Trp6 channels by this agent. It is possible that mAChRs activate an unknown calcium entry pathway via PI3K. However, this is less likely because another structurally unrelated PI3K inhibitor, wortmannin, did not show similar inhibitory effects.

The results of the present study indicated that the PI3K inhibitor LY294002 does not affect IP₃-dependent calcium release, but does suppress mAChR-induced calcium entry in PC12h cells. These observations represent direct evidence that PI3K does not interfere with PLC-dependent calcium release, which is supported by the lack of PI3K-activating stimuli that inhibit PLC-dependent cellular responses. The suppressive effect of LY294002 on calcium entry is a novel finding and has significant implication for previous and future usage of this agent. Recent reports on the effects of LY294002 on wortmannin-insensitive and PI3K-independent cellular

processes, including apoptosis and immediate early gene expression (32, 33), should be reconsidered based on the present results because cellular calcium is an important factor in these processes. In addition, the present results indicate that careful interpretation of the results obtained with LY294002 and appropriate comparisons with wortmannin are necessary.

Acknowledgments

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