Activation of PLC-δ1 by G_{i/o}-coupled receptor agonists

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Submitted 25 May 2004; accepted in final form 1 August 2004

Murthy, Karnam S., Huiping Zhou, Jiean Huang, and Srinivas N. Pentyala. Activation of PLC-δ1 by G_{i/o}-coupled receptor agonists. Am J Physiol Cell Physiol 287: C1679–C1687, 2004; doi:10.1152/ajpcell.00257.2004.—The mechanism of phospholipase (PLC)-δ activation by G protein-coupled receptor agonists was examined in rabbit gastric smooth muscle. Ca^{2+} stimulated an eightfold increase in PLC-δ1 activity in permeabilized muscle cells. Treatment of dispersed or cultured muscle cells with three G_{i/o}-coupled receptor agonists (somatostatin, δ-opioid agonist [D-Pen²,D-Pen⁵]enkephalin, and A₁ agonist cyclopentyl adenosine) caused delayed increase in phosphoinositide (PI) hydrolysis (8- to 10-fold) that was strongly inhibited by PIP2 (50% and strongly inhibited by SKF-96365, a blocker of store-operated Ca^{2+} channels. Treatment of the cells with a G_{i/o}-coupled receptor agonist, CCK-8, caused only transient, PLC-β1-mediated PI hydrolysis. Unlike G_{i/o}-coupled receptor agonists, CCK-8 activated RhoA and stimulated RhoA:PLC-δ1 association. Inhibition of RhoA activity with C3 exoenzyme or by overexpression of dominant-negative RhoA(T19N) or G_{13} miogene unmasked a delayed increase in PI hydrolysis that was strongly inhibited by coexpression of PLC-δ1(E341R/D343R) or SKF-96365. Agonist-independent capacitative Ca^{2+} influx and was not observed in the absence of extracellular Ca^{2+}, but was partly inhibited by nifedipine (16–30%) and strongly inhibited by SKF-96365, a blocker of store-operated Ca^{2+} channels. Treatment of the cells with a G_{i/o}-coupled receptor agonist, CCK-8, caused only transient, PLC-β1-mediated PI hydrolysis. Unlike G_{i/o}-coupled receptor agonists, CCK-8 activated RhoA and stimulated RhoA:PLC-δ1 association. Inhibition of RhoA activity with C3 exoenzyme or by overexpression of dominant-negative RhoA(T19N) or G_{13} miogene unmasked a delayed increase in PI hydrolysis that was strongly inhibited by coexpression of PLC-δ1(E341R/D343R) or SKF-96365. Agonist-independent capacitative Ca^{2+} influx induced by thapsigargin stimulated PI hydrolysis (8-fold), which was partly inhibited by nifedipine (~25%) and strongly inhibited by SKF-96365 (~75%) and in cells expressing PLC-δ1(E341R/D343R). Agonist-independent Ca^{2+} release or Ca^{2+} influx via voltage-gated Ca^{2+} channels stimulated only moderate PI hydrolysis (2- to 3-fold), which was abolished by PLC-δ1 antibody or nifedipine. We conclude that PLC-δ1 is activated by G_{i/o}-coupled receptor agonists that do not activate RhoA. The activation is preferentially mediated by Ca^{2+} influx via store-operated Ca^{2+} channels.

phospholipase C; G protein

FOUR FAMILIES OF phosphoinositide-specific phospholipases (PI-PLC), containing a total of eleven distinct isozymes (PLC-δ1, -β2, -β3, -β4; PLC-γ1, -γ2; PLC-δ1, -δ2, -δ3, -δ4; and PLC-ɛ) have been cloned and characterized (13, 19, 41, 42). All the isozymes contain an NH2-terminal pleckstrin homology (PH) domain for protein-protein interaction, a central catalytic domain with several highly conserved residues (His³¹¹, His³⁵⁶, and Glu⁴¹⁴), and a COOH-terminal, Ca^{2+}-dependent, membrane-targeting C2 domain (10–13, 16, 42). A long COOH-terminal tail is a characteristic feature of PLC-δ isozymes and mediates Go-dependent binding and activation of PLC-δ1 (13, 22, 42, 46). PLC-β2 and PLC-β3 are activated via binding of their PH domains to G_{βγ} subunits, particularly G_{βγ}5 (5, 6, 20, 29, 31, 33, 39). PLC-ɛ contains two COOH-terminal Ras-associating domains and a Ras guanine nucleotide exchange factor motif and is variously activated by Ras, Rap, G_{α12}, G_{βγ}, and RhoA (27, 47). PLC-γ isozymes are activated by tyrosine phosphorylation of two conserved residues (Tyr⁷⁷¹ and Tyr¹⁸³) via receptor tyrosine kinases (13, 42).

The PH domain is essential for activation of PLC-δ, and its deletion abolishes PLC-δ activity (2, 8, 15). The PH domain of PLC-δ binds phosphatidylinositol 4,5-bisphosphate (PIP₂), which anchors the enzyme to the plasma membrane and enhances its catalytic activity (15). Although the PH domain of PLC-δ binding is too weak to induce activation of PLC-δ (15, 44). Four specific receptors (α₁B and α₁DAD, α-thromboxane receptors, and oxytocin receptors) can activate PLC-δ via coupling to the atypical G protein (Goα), also known as transglutaminase-II (3, 18, 40). The mechanism of PLC-δ activation via Goα is not known. A recent study suggests that PLC-δ is activated by hypotonicity in some neurons; the effect is mediated by neuromodulin, a neuronal, membrane-anchored osmosensory protein, but the mechanism has not been determined (7).

PLC-δ1, the most abundant and widely expressed isoform of PLC-δ, is typically activated by Ca^{2+} in the range of 0.1–10 μM (1, 26, 28). Despite its sensitivity to Ca^{2+} (~100-fold higher than that of PLC-β or PLC-γ), PLC-δ is not activated by agonists that mobilize intracellular Ca^{2+}, possibly because PLC-δ is inactivated by PLC-β-dependent generation of protein kinase C (PKC) and inositol triphosphate (IP3); the latter competes with PIP2 for binding to PLC-δ (1, 4). Ca^{2+} influx induced by Ca^{2+} ionophores or via voltage-gated Ca^{2+} channels (e.g., depolarizing concentrations of KCl) causes moderate PI hydrolysis, but the specific isozyme mediating this effect has not been identified (21, 43). In a recent study, activation of PLC-δ by capacitative Ca^{2+} influx induced by thapsigargin or bradykinin was observed only after overexpression of PLC-δ (23).

In this study, we have identified a unique mechanism for activation of PLC-δ1 via G_{i/o}-coupled receptors. The inability of G_{i/o}-coupled receptors to activate PLC-δ1 reflected concurrent activation of RhoA.

EXPERIMENTAL PROCEDURES

Preparation of dispersed and cultured muscle cells. Smooth muscle cells were isolated from the circular muscle layer of rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation, as described previously (32, 36–38). In some experiments, cells were also isolated from the circular or longitudinal muscle layers of small intestine. The cells were resuspended in enzyme-free medium con-
sisting of 120 mM NaCl, 4 mM KCl, 2.6 mM KH₂PO₄, 2 mM CaCl₂, 0.6 mM MgCl₂, 25 mM HEPES, 14 mM glucose, and 2.1% Eagle’s essential amino acid mixture. The cells were harvested by filtration through 500-μm Nitex mesh and centrifuged twice at 350 g for 10 min. In experiments with blocking antibodies, the cells were permeabilized with saponin (35 μg/ml) in a medium containing 100 mM Ca²⁺ and resuspended in 1.5 mM ATP and an ATP-regenerating system (5 mM creatine phosphate and 10 U/ml creatine phosphokinase). In some experiments, the muscle cells were cultured in DMEM containing 10% FBS until they attained confluence and were used in the first passage.

Assay of PLC activity. Inositol phosphate formation was measured as described previously using anion exchange chromatography (29, 31–33). Measurements were made in the absence of Li³⁺. Freshly dispersed muscle cells (10⁶ cells/ml) were labeled with myo-[³H]inositol for 3 h and cultured muscle cells for 24 h. The cells were then centrifuged at 350 g for 10 min to remove excess [³H]inositol and resuspended in 10 ml fresh HEPES medium. The reaction was terminated by the addition of 940 μl chloroform-methanol-HCl (50:100:1). The samples were extracted with 340 μl chloroform and 340 μl H₂O and centrifuged at 1,000 g for 15 min. The upper aqueous phase was applied to a DOWEX AG-1 column, and [³H]inositol phosphates were eluted with 0.8 M ammonium formamate-0.1 M formic acid. Radioactivity was determined by liquid scintillation and expressed as counts per minute per milligram of protein.

Expression of Gα₁₃ minigene, dominant-negative PLC-δ1 and RhoA, and constitutively active RhoA in cultured smooth muscle cells. Gα₁₃ minigene (MGLHDNLKQLMLQ), dominant-negative PLC-δ1 (E341R/D343R) and RhoA(T19N), and constitutively active PLC-δ1 and PLC-δ1(E341R/D343R) and RhoA(T19N), and constitutively active RhoA cDNA were a gift of Dr. Andrea Todisco (University of Michigan).

RESULTS

Activation of PLC-δ1 by Ca²⁺. Ca²⁺ concentrations in the range 0.1–1.0 μM stimulated PI hydrolysis (PLC activity) in permeabilized gastric smooth muscle cells in a concentration-dependent fashion [maximal 8- to 10-fold increase in PLC activity with 1 μM Ca²⁺ (5,021 ± 468 cpm/mg protein above basal); Fig. 1A]. The isoform of PLC activated by Ca²⁺ in smooth muscle was identified by functional blockade with specific antibodies. Our previous studies had shown that all PI-specific PLC isozymes, including PLC-β1, -2, -3, and -4, PLC-γ1, and PLC-δ1, were expressed in gastrointestinal smooth muscle (29, 32). Preincubation of permeabilized muscle cells for 1 h with PLC-δ1 antibody (10 μg/ml) inhibited various isoforms of PLC-β were from Santa Cruz Biotechnology (Santa Cruz, CA); pGreen Lantern-1 and Lipofectamine Plus reagent were from Life Technologies GIBCO-BRL (Rockville, MD); SKF-96365 was from Biomol (Plymouth Meeting, PA); and all other reagents were from Sigma. RhoA CDNA was a gift of Dr. Andrea Todisco (University of Michigan).
maximal Ca\(^{2+}\)-stimulated PLC activity by 84 ± 2%, whereas preincubation with PLC-β1, PLC-β2, PLC-β3, PLC-β4, or PLC-γ1 antibody had no effect (Fig. 1B). The pattern of inhibition by PLC antibodies implied that Ca\(^{2+}\) specifically activated PLC-61.

Dual activation of PLC-β3 and PLC-61 by G\(_{i/o}\)-coupled receptors. Previous studies in these smooth muscle cells had shown that G\(_{i/o}\)-coupled receptor agonists elicit an initial, transient (~2 min) increase in PI hydrolysis mediated by G\(_{i/o}\)-dependent activation of PLC-β3 (29, 31, 33, 36). As shown in Fig. 2, treatment of cultured smooth muscle cells with a G\(_{i,2}\)-coupled receptor agonist ([D-Pen\(^2\),D-Pen\(^5\)]enkephalin or DPDPE) caused an initial PLC-β3-dependent increase in PI hydrolysis followed by a delayed increase (~8-fold above basal levels). The delayed increase in PI hydrolysis was virtually abolished in cells expressing dominant-negative PLC-61(E341R/D343R), whereas the initial increase was not affected. The delayed increase in PI hydrolysis was also inhibited by expression of a constitutive active RhoA. The significance of this observation is discussed subsequently.

A similar 8- to 10-fold increase in PI hydrolysis was elicited by G\(_{i,1}\)- and G\(_{i,3}\)-coupled receptor agonists [somatostatin and the A\(_1\) agonist cyclopentyl adenosine (CPA), respectively; Fig. 3A]. The increase induced by these agonists was strongly inhibited in cells expressing PLC-61(E341R/D343R) (Fig. 3A). The delayed increase in PI hydrolysis was not observed in the absence of extracellular Ca\(^{2+}\) and was virtually abolished by SKF-96365, an inhibitor of store-operated and voltage-gated Ca\(^{2+}\) influx. In some experiments, the cells were incubated with 1 mM SKF-96365 for 10 min before addition of Ca\(^{2+}\)\(^{2+}\). B: freshly dispersed muscle cells labeled with myo-[\(^{3}\)H]inositol for 24 h. Cells were incubated for 5 min in the absence of extracellular Ca\(^{2+}\) with the G\(_{i/o}\)-coupled receptor agonist somatostatin (SST), DPDPE, or cyclopentyl adenosine (CPA; 1 μM each) to deplete Ca\(^{2+}\) stores. Ca\(^{2+}\) (2 mM) was then added to elicit capacitative Ca\(^{2+}\) influx. In some experiments, the cells were incubated with 1 mM SKF-96365 for 10 min before addition of Ca\(^{2+}\). C: Western blot analysis of RhoA and PLC-61 in cells expressing dominant-negative PLC-61(E341R/D343R) (lane 2). Values are means ± SE of 3 experiments.

Fig. 3. Activation of PLC-61 by G\(_{i/o}\)-coupled receptor agonists. A: cultured muscle cells expressing dominant-negative PLC-61(E341R/D343R) or vector alone were labeled with myo-[\(^{3}\)H]inositol for 24 h. Cells were incubated for 5 min in the absence of extracellular Ca\(^{2+}\) with the G\(_{i/o}\)-coupled receptor agonist somatostatin (SST), DPDPE, or cyclopentyl adenosine (CPA; 1 μM each) to deplete Ca\(^{2+}\) stores. Ca\(^{2+}\) (2 mM) was then added to elicit capacitative Ca\(^{2+}\) influx. In some experiments, the cells were incubated with 1 mM SKF-96365 for 10 min before addition of Ca\(^{2+}\). B: freshly dispersed muscle cells labeled with myo-[\(^{3}\)H]inositol were incubated for 5 min in the absence of extracellular Ca\(^{2+}\) with the G\(_{i/o}\)-coupled receptor agonists somatostatin, DPDPE, or CPA (1 μM each) to deplete Ca\(^{2+}\) stores. Ca\(^{2+}\) (2 mM) was then added to elicit capacitative Ca\(^{2+}\) influx. In some experiments, the cells were incubated with the store-operated Ca\(^{2+}\) channel blocker SKF-96365 (1 μM) or with voltage-gated Ca\(^{2+}\) channel blocker nifedipine (1 μM) for 10 min before addition of Ca\(^{2+}\). PI hydrolysis was expressed as total inositol phosphates (cpm/mg protein). Agonist-induced PI hydrolysis at 5 min was strongly inhibited in cells expressing PLC-61(E341R/D343R) or by SKF-96365 and slightly inhibited by nifedipine. Inset: Western blot analysis of RhoA and PLC-61 in cells expressing vector alone (lane 1) and overexpressing PLC-61(E341R/D343R) (lane 2). Values are means ± SE of 3 experiments.
The agonists caused a delayed 8- to 10-fold increase in PI hydrolysis similar in magnitude to that induced by 1 μM Ca²⁺ in permeabilized muscles. The response was not observed in the absence of extracellular Ca²⁺ (data not shown), was virtually abolished by SKF-96365, and was only slightly inhibited by nifedipine (16–30%), implying that PI hydrolysis was dependent on capacitative Ca²⁺ influx via store-operated Ca²⁺ channels and, to a lesser extent, via voltage-gated Ca²⁺ channels (Fig. 3B).

Selective activation of PLC-β by Gq-coupled receptors. Treatment of dispersed muscle cells with CCK-8 elicited a transient stimulation of PI hydrolysis (Fig. 4A). The initial increase was not followed by a delayed increase in PI hydrolysis, was inhibited by 83 ± 5% in muscle cells preincubated for 60 min with PLC-β1 antibody (10 μg/ml), but was not affected in cells preincubated with PLC-γ1 or PLC-δ1 antibody (Fig. 4B). Earlier studies had shown that CCK-stimulated PI hydrolysis in permeabilized muscle cells was blocked by incubation with Goα antibody (32). The effectiveness of Go protein and PLC-β antibodies in blocking agonist-induced, Go protein-dependent PI hydrolysis in permeabilized smooth muscle was characterized in previous studies (29, 31–34, 36).

Suppression of PLC-δ1 stimulation by activated RhoA. We postulated that the difference in the ability of Goα- and Gq-coupled receptor agonists to elicit activation of PLC-δ1 could reflect the difference in their ability to stimulate RhoA activity. Previous studies in smooth muscle cells had shown that Gq-coupled receptors (e.g., CCK₄₃, m₃) that activate PLC-β1 also activate Go₁₃ and RhoA (37, 38). In contrast, Gi-coupled receptors (somatostatin receptor 3, δ-opioid, A₁, m₂) that activate PLC-β3 also activate Cdc42/Rac1 and PI 3-kinase but not Go₁₃ or RhoA (36, 48).

CCK-8 induced RhoA:PLC-δ1 association (Fig. 5A) and stimulated RhoA activity in a time-dependent fashion (Fig. 5B). In contrast, somatostatin, DPDPE, and CPA did not stimulate RhoA activity (Fig. 5C). Treatment of freshly dispersed muscle cells with the RhoA inhibitor, C3 exoenzyme, inhibited CCK-induced RhoA:PLC-δ1 association (Fig. 5A) and RhoA activity (Fig. 5C) and unmasked a delayed increase in PI hydrolysis (Fig. 6). The initial increase measured in the first minute and mediated by PLC-β1 as shown in Fig. 4B was not affected by C3 exoenzyme. The delayed increase (measured 5 min after addition of CCK-8) coincided with the period of capacitative Ca²⁺ influx and was not observed in the absence of extracellular Ca²⁺. Neither the initial nor the delayed increase in PI hydrolysis was affected by the PKC inhibitor bisindolylmaleimide (1 μM) or the PK kinase inhibitor Y-27632 (1 μM; data not shown).

A delayed increase in PI hydrolysis induced by CCK-8 was also unmasked in cultured smooth muscle cells expressing dominant-negative RhoA (T19N; Fig. 7A) or Go₁₃ minigene (Fig. 7B). The delayed PI hydrolysis caused by CCK-8 reflected activation of PLC-δ1, since it could not be elicited in cultured smooth muscle cells expressing coexpressing RhoA(T19N) and PLC-δ1(E341R/D343R) or coexpressing Go₁₃ minigene and PLC-δ1(E341R/D343R) (Fig. 7, A and B). Expression of Go₁₃ minigene inhibited RhoA:PLC-δ1 association (Fig. 8A) and RhoA activity (Fig. 8B). The early phase of PI hydrolysis mediated by PLC-β1 was not affected by expression of RhoA(T19N), Go₁₃ minigene, or PLC-δ1(E341R/D343R) (data not shown).

Further evidence that association of PLC-δ1 with activated RhoA prevents activation of PLC-δ1 is provided in Fig. 2, where the delayed increase in PI hydrolysis induced by the Gi-coupled receptor agonist DPDPE was virtually abolished in cells expressing a constitutively active RhoA(G14V).

Activation of PLC-δ1 by agonist-independent capacitative Ca²⁺ influx. The relative importance of Ca²⁺ influx via store-operated and voltage-gated Ca²⁺ channels in stimulating PLC-δ1 activity was examined further using thapsigargin and KCl to activate preferentially store-operated and voltage-gated Ca²⁺ channels, respectively. Treatment of muscle cells for 20 min with thapsigargin in the absence of Ca²⁺ followed by addition of 2 mM Ca²⁺ caused an eightfold increase in PI hydrolysis, similar in magnitude to that elicited by Gi-coupled receptor agonists (Fig. 9A). No increase in PI hydrolysis was observed in the absence of extracellular Ca²⁺ (data not shown). PI hydrolysis induced by thapsigargin was inhibited strongly (82 ± 6%) by SKF-96365 (Fig. 9A) and minimally by nifedipine (13 ± 2%). PI hydrolysis induced by thapsigargin in cultured muscle cells (4,039 ± 479 cpm/mg protein) was strongly inhibited (75 ± 5%) in cells overexpressing a PLC-δ1(E341R/D343R) mutant (Fig. 9B), implying that it was mediated by PLC-δ1.
In contrast, Ca\textsuperscript{2+} influx induced by treatment of intact muscle cells with a depolarizing concentration of KCl (20 mM) or with \(\alpha,\beta\)-methylene ATP, a P2X receptor agonist, caused only a moderate two- to threefold increase in PI hydrolysis that was abolished by nifedipine (Fig. 10A). Previous studies in these cells had shown that activation of the P2X receptor, a ligand-gated cationic channel, depolarizes smooth muscle cells and stimulates Ca\textsuperscript{2+} influx via voltage-gated Ca\textsuperscript{2+} channels (34).

Activation of PLC-\(\delta 1\) by agonist-independent Ca\textsuperscript{2+} release.

As noted above, PI hydrolysis induced by CCK-8 in permeabilized muscle cells was not affected by preincubation with PLC-\(\delta 1\) antibody (Fig. 4), suggesting either that Ca\textsuperscript{2+} release did not activate PLC-\(\delta 1\) or that activation of PLC-\(\delta 1\) was offset by concomitant inhibition by IP3 and PKC. In the absence of agonist, treatment of permeabilized intestinal circular muscle cells with IP3 (1 \(\mu M\)) caused a significant twofold increase in PI hydrolysis that was abolished in cells preincubated for 60 min with PLC-\(\delta 1\) antibody (Fig. 10B). It is possible that the response to Ca\textsuperscript{2+} release induced by IP3 might have been attenuated by the inhibitory effect of IP3 on PLC-\(\delta 1\) activity. To obviate the confounding effect of IP3, the experiments were repeated in intestinal longitudinal muscle cells, which express predominantly IP3-insensitive, ryanodine receptors/Ca\textsuperscript{2+} channels that are highly sensitive to Ca\textsuperscript{2+} and cADP ribose (24, 25). Treatment of permeabilized intestinal longitudinal muscle cells with cADP ribose caused a somewhat higher threefold increase in PI hydrolysis, which was inhibited by 73 \(\pm\) 5% in cells preincubated with PLC-\(\delta 1\) antibody.

The pattern that emerges from comparison of PLC-\(\delta 1\) activity induced by \(G_{i/o}\)-dependent or -independent capacitative Ca\textsuperscript{2+} influx with PLC-\(\delta 1\) activity induced by \(G\) protein-independent Ca\textsuperscript{2+} influx (KCl; \(\alpha,\beta\)-methylene ATP) or Ca\textsuperscript{2+} release (IP3 and cADP ribose) is that the mechanism of Ca\textsuperscript{2+} entry rather than the magnitude of Ca\textsuperscript{2+} entry or Ca\textsuperscript{2+} release is the main determinant of PLC-\(\delta 1\) activity.

**DISCUSSION**

This study provides the first evidence that \(G_{i/o}\)-coupled receptors can activate PLC-\(\delta 1\) and that activation is preferentially mediated by capacitative Ca\textsuperscript{2+} influx. The study also shows that the inability of \(G_{i/o}\)-coupled receptors to activate PLC-\(\delta 1\) reflects concurrent activation of RhoA, which inactivates PLC-\(\delta 1\) by binding directly to RhoA or indirectly via RhoGAP (17). A link between PI-PLC activity and capacitative Ca\textsuperscript{2+} influx was suggested by earlier studies showing an

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**Fig. 5.**Selective activation of RhoA and stimulation of RhoA:PLC-\(\delta 1\) association by \(G_{i/o}\)-coupled receptor agonist. A: freshly dispersed muscle cells were treated for 5 min with various agonists [CCK-8, somatostatin, \(\delta\)-opioid agonist DPDPDE (\(\delta\)), and CPA]. Muscle cells were lysed and treated with PLC-\(\delta 1\) antibody for 12 h to obtain PLC-\(\delta 1\) immunoprecipitates (IP). PLC-\(\delta 1\) immunoprecipitates were separated by SDS-PAGE and probed with RhoA antibody to examine the association of PLC-\(\delta 1\):RhoA. B: freshly dispersed smooth muscle cells were treated with CCK-8 (1 nM) for various intervals. Muscle cell lysates were incubated with glutathione-agarose slurry of Rho-kinase, and GTP-bound RhoA (RhoA activity) was analyzed by SDS-PAGE followed by Western blot analysis. C: freshly dispersed muscle cells were treated with various agonists [CCK-8, somatostatin, \(\delta\)-opioid agonist DPDPDE (\(\delta\)), and CPA] for 5 min, and RhoA activity was determined. In some experiments, the cells were preincubated for 3 h with the RhoA inhibitor C3 exoenzyme (C3E). CCK, but not somatostatin, DPDPDE, or CPA, stimulated RhoA activity and induced association of PLC-\(\delta 1\):RhoA. CCK-induced RhoA activity and association of PLC-\(\delta 1\):RhoA were blocked by C3 exoenzyme. In all experiments, maximally effective concentrations were used (1 nM for CCK-8 and 1 \(\mu M\) for other agonists). Values are means \(\pm\) SE of 4–6 experiments.

**Fig. 6.** Activation of PLC-\(\delta 1\) by \(G_{i/o}\)-coupled receptor agonists after inactivation of RhoA. Freshly dispersed muscle cells labeled with myo-[\(3^H\)]inositol were treated with CCK-8 (1 nM) for 1 or 5 min in the absence of extracellular Ca\textsuperscript{2+}. Capacitative Ca\textsuperscript{2+} influx was elicited by the addition of 2 mM Ca\textsuperscript{2+} at 5 min. PI hydrolysis was measured using anion exchange chromatography and expressed as total inositol phosphates (cpm/mg protein). In some experiments, freshly dispersed muscle cells were preincubated for 3 h with the RhoA inhibitor C3 exoenzyme. Inhibition of RhoA had no effect on CCK-induced initial increase in PI hydrolysis but unmasked a delayed increase in PI hydrolysis. Values are means \(\pm\) SE of 4 experiments.
increase in PI-PLC activity in cells overexpressing PLC-δ1, but the specific requirements of coupling to Gi/o and absence of RhoA activity were not addressed (23).

Previous studies in smooth muscle cells had shown that G_{i/o}-coupled receptor agonists, including all three agonists used in this study, cause an initial G_{i/o}-dependent stimulation of PLC-β3 activity, resulting in IP_{3}-dependent Ca^{2+} release and contraction (25, 29, 31, 33). Here we show that these agonists also induce a delayed PI hydrolysis that is virtually abolished in smooth muscle cells expressing a dominant-negative PLC-δ1(E341R/D343R) or a constitutively active RhoA(G14V), implying that PI hydrolysis was mediated by PLC-δ1 and blocked by activated RhoA. Conversely, G_{q/13}-coupled agonists that activate RhoA did not cause delayed PI hydrolysis. Inactivation of RhoA with C3 exoenzyme or by expression of dominant-negative RhoA(T19N) unmasked a delayed PI hydrolysis mediated by PLC-δ1. Coexpression of dominant-negative RhoA(T19N) or G_{q/13} minigene with PLC-δ1 suppressed delayed PI hydrolysis, implying that it was mediated by PLC-δ1.

The initial increase in PI hydrolysis resulting from G_{q/13}-dependent activation of PLC-δ1 or G_{q/13}-dependent activation of PLC-β3 induces IP_{3}-dependent Ca^{2+} release followed by capacitative Ca^{2+} influx triggered by depletion of Ca^{2+} stores. Here we show that capacitative Ca^{2+} influx is the proximate stimulus of PLC-δ1 activity. Delayed PI hydrolysis initiated by G_{q/13}-coupled receptor agonists (or by G_{q/13}-coupled agonists after inactivation of RhoA) was not observed in the absence of extracellular Ca^{2+}, was only slightly decreased by blockade of voltage-gated Ca^{2+} channels with nifedipine, but was virtually
aborted by blockade of both voltage-gated and store-operated Ca\(^{2+}\) channels with SKF-96365. The importance of Ca\(^{2+}\) influx via store-operated channels was evident in the relative effects of thapsigargin, KCl, and α,β-methylene ATP. Ca\(^{2+}\) influx via voltage-gated Ca\(^{2+}\) channels induced by depolarizing concentrations of KCl or by α,β-methylene ATP caused only a moderate increase in PLC-δ1 activity, whereas Ca\(^{2+}\) influx via store-operated Ca\(^{2+}\) channels, induced by depletion of Ca\(^{2+}\) stores with thapsigargin, caused a maximal increase in PLC-δ1 activity that was abolished by SKF-96365. PI hydrolysis induced by thapsigargin was virtually abolished in cells expressing PLC-δ1(E341R/D343R), corroborating the identity of the PLC isozyme activated by capacitative Ca\(^{2+}\) influx.

The profound effect on PLC-δ1 activity of Ca\(^{2+}\) influx via store-operated Ca\(^{2+}\) channels (inhibition by SKF-96365) relative to that of Ca\(^{2+}\) influx via voltage-gated Ca\(^{2+}\) channels (inhibition by nifedipine) or to that of Ca\(^{2+}\) release suggests a close association of PLC-δ1 and store-operated Ca\(^{2+}\) channels. A similar association has been observed between these channels and membrane-bound Ca\(^{2+}\)-sensitive adenyl cyclases that leads to inhibition of adenyl cyclase type V/VI and activation of adenyl cyclase type VIII (9, 14, 35). Preliminary studies show association of PLC-δ1, adenyl cyclase V/VI, A-kinase anchoring protein, and activated RhoA with caveolin that could act as a membrane-bound scaffolding protein (Murthy KS, unpublished observations). Recent studies suggest an association between caveolin and Trp4 channels involved in capacitative Ca\(^{2+}\) influx (45).

The exquisite sensitivity of PLC-δ1 to Ca\(^{2+}\) relative to the sensitivity of PLC-β1 or PLC-γ1 previously demonstrated by in vitro assay (1) was corroborated in vivo in this study (Fig. 9)

![Graph](http://ajpcell.physiology.org/)

**Fig. 9.** Activation of PLC-δ1 by agonist-independent capacitative Ca\(^{2+}\) influx. Freshly dispersed muscle cells (A) and cultured muscle cells expressing vector alone or overexpressing dominant-negative PLC-δ1(E341R/D343R) (B) were incubated with thapsigargin (TG, 2 μM) in the absence of extracellular Ca\(^{2+}\) for 30 min to deplete intracellular Ca\(^{2+}\); 2 mM Ca\(^{2+}\) were then added to elicit capacitative Ca\(^{2+}\) influx. In some experiments, the cells were incubated with the store-operated Ca\(^{2+}\) channel blocker SKF-96365 (SKF, 1 μM) for 10 min before addition of Ca\(^{2+}\). PI hydrolysis was measured using anion exchange chromatography and expressed as total inositol phosphates (cpm/mg protein) in cells prelabelled with [\(^{3}\)H]inositol. Thapsigargin-induced PI hydrolysis was inhibited by SKF-96365 or in cells expressing PLC-δ1 (E341R/D343R). *Inset:* Western blot analysis in cells expressing vector alone (lane 1) or overexpressing PLC-δ1 (E341R/D343R) (lane 2). Values are means ± SE of 3 experiments. **Significant inhibition \((P < 0.001)\) of PI hydrolysis by SKF-93635. ***Significant inhibition \((P < 0.001)\) of PI hydrolysis in cells overexpressing PLC-δ1(E341R/D343R).

![Graph](http://ajpcell.physiology.org/)

**Fig. 10.** Activation of PLC-δ1 by Ca\(^{2+}\) release and Ca\(^{2+}\) influx. A: dispersed muscle cells labeled with [\(^{3}\)H]inositol were treated for 1 min with the ligand-gated P2X receptor agonist α,β-methylene ATP (1 μM) or with 20 mM KCl for 1 min in the presence or absence of the Ca\(^{2+}\) channel blocker nifedipine (1 μM). B: dispersed intestinal muscle cells labeled with [\(^{3}\)H]inositol were permeabilized and then treated with 1 μM inositol trisphosphate (IP\(_3\), circular muscle cells) or 1 μM cADPR ribose (longitudinal muscle cells) for 30 s to elicit Ca\(^{2+}\) release. In some experiments, muscle cells were preincubated with PLC-δ1 antibody (10 μg/ml) for 1 h. PI hydrolysis was expressed as cpm/mg protein of total inositol phosphates. Values are means ± SE of 3–6 experiments. **Significant inhibition \((P < 0.01)\) of PI hydrolysis by PLC-δ1 antibody or nifedipine.
1. Even Ca\(^{2+}\) release induced by exogenous IP\(_3\) or cADP ribose caused a moderate increase in PLC-\(\delta\)1 activity. The identity of the PLC isozyme activated by Ca\(^{2+}\) release was confirmed by blockade with PLC-\(\delta\)1 antibody. A potential increase in PLC-\(\delta\)1 activity that might result from agonist-induced Ca\(^{2+}\) release would be offset by concurrent inhibition of PLC-\(\delta\)1 by IP\(_3\) and PKC.

Although this study identifies a physiological mechanism for activation of PLC-\(\delta\)1 by G\(_{\text{q}}\)-coupled receptor agonists, the functional role of PLC-\(\delta\)1 and delayed PI hydrolysis has yet to be elucidated. Conceivably, delayed generation of IP\(_3\) could alter the dynamics of Ca\(^{2+}\) and Kidney Diseases Grant DK-15564.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-15564.

REFERENCES


