P2Y₂ Purinergic and M₃ Muscarinic Acetylcholine Receptors Activate Different Phospholipase C-β Isoforms That Are Uniquely Susceptible to Protein Kinase C-dependent Phosphorylation and Inactivation*

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Activation of phospholipase C-β (PLC-β) by G protein-coupled receptors typically results in rapid but transient second messenger generation. Although PLC-β deactivation may contribute to the transient nature of this response, the mechanisms governing PLC-β deactivation are poorly characterized. We investigated the involvement of protein kinase C (PKC) in the termination of PLC-β activation induced by endogenous P2Y₂ purinergic receptors and transfected M₃ muscarinic acetylcholine receptors (mAChR) in Chinese hamster ovary cells. Activation of P2Y₂ receptors causes Gαq₁₁ to associate with PLC-β₃, whereas M₃ mAChR activation causes Gαq₁₁ to associate with both PLC-β₁ and PLC-β₃ in these cells. Phosphorylation of PLC-β₃, but not PLC-β₁, is induced by activating either P2Y₂ receptors or M₃ mAChR. We demonstrate that PKC rather than protein kinase A mediates the G protein-coupled receptor-induced phosphorylation of PLC-β₃. The PKC-mediated phosphorylation of PLC-β₃ diminishes the interaction of Gαq₁₁ with PLC-β₃, thereby contributing to the termination of PLC-β₃ activity. These findings indicate that the distinct temporal profiles of PLC activation by P2Y₂ receptors and mAChR may arise from the differential activation of PLC-β₁ and PLC-β₃ by the receptors, coupled with a selective PKC-mediated negative feedback mechanism that targets PLC-β₃ but not PLC-β₁.

Phospholipase C-β (PLC-β) is a key signal transduction enzyme catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield the second messengers inositol 1,4,5-trisphosphate (IP₃) and 1,2-bisphosphate to yield the second messengers inositol 1,4,5-trisphosphate (IP₃) and 1,2-bisphosphate. These messengers activate PLC-β₃ and PLC-β₁. However, the mechanisms governing PLC-β₁ and PLC-β₃ deactivation are poorly characterized. We investigated the involvement of protein kinase C (PKC) in the termination of PLC-β₁ and PLC-β₃ activation induced by endogenous P2Y₂ purinergic receptors and transfected M₃ mAChR in Chinese hamster ovary cells. Activation of P2Y₂ receptors causes Gαq₁₁ to associate with PLC-β₃, whereas M₃ mAChR activation causes Gαq₁₁ to associate with both PLC-β₁ and PLC-β₃ in these cells. Phosphorylation of PLC-β₃, but not PLC-β₁, is induced by activating either P2Y₂ receptors or M₃ mAChR. We demonstrate that PKC rather than protein kinase A mediates the G protein-coupled receptor-induced phosphorylation of PLC-β₃. The PKC-mediated phosphorylation of PLC-β₃ diminishes the interaction of Gαq₁₁ with PLC-β₃, thereby contributing to the termination of PLC-β₁ and PLC-β₃ activity in these cells. These findings indicate that the distinct temporal profiles of PLC activation by P2Y₂ receptors and mAChR may arise from the differential activation of PLC-β₁ and PLC-β₃ by the receptors, coupled with a selective PKC-mediated negative feedback mechanism that targets PLC-β₃ but not PLC-β₁.

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The abbreviations used are: PLC-β, phospholipase C-β; BIM, bisindolylmaleimide I; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; GTP-γS, guanosine 5'-O-(3-thiotriphosphate); IP₃, inositol (1,4,5)-trisphosphate; mAChR, muscarinic acetylcholine receptor; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
tion make it difficult to determine unambiguously where PKC acts to limit PLC-β activity.

The purpose of this study was to determine the role of PKC in the poorly characterized termination of GPCR-stimulated PLC activity. We investigated PLC-β activity regulated by endogenous P2Y2 purerergic receptors (25) and transfected M2 muscarinic acetylcholine receptors (mACHR) (11) expressed in Chinese hamster ovary (CHO) cells. It was previously reported that P2Y2 receptors, like most GPCRs, transiently activate PLC-β (25), whereas M2 mACHR activate PLC-β for periods longer than 10 min (6, 9, 11). We found that P2Y2 receptors functionally couple only to PLC-β3, whereas M2 mACHR functionally couple to both PLC-β1 and PLC-β3 in CHO cells. Activating P2Y2 receptors or M2 mACHR induces the phosphorylation of PLC-β3, but not PLC-β1, in these cells. We demonstrate that PKC, rather than PKA, mediates the GPCR-induced phosphorylation of PLC-β3. Thus, PLC-β3 phosphorylation does not result from cross-regulation by adenyl cyclase acting on PLC signaling. Our results indicate that PKC participates in a negative feedback loop to inhibit the interaction of Gαq/11 with PLC-β3, limiting the duration of PLC-β3 activity. This PKC-mediated negative regulation of PLC-β3, coupled with the differential activation of PLC-β1 and -β3 by M2 mACHR and P2Y2 receptors, offers a mechanism for the distinct temporal profiles of PLC activation by these two receptors.

EXPERIMENTAL PROCEDURES

**Reagents**—Rabbit polyclonal antibodies to Gαq/11, PLC-β1, or PLC-β3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled anti-rabbit antibodies, ECL reagents, and [35S]GTPγS (specific activity, 1030 Ci/mmol) were purchased from Amersham Pharmacia Biotech. [32P]Orthophosphate (specific activity, 74 Ci/mmol) and [35S]Cysteine/methionine (specific activity, 1200 Ci/mmol) were purchased from ICN (Costa Mesa, CA). The fluorescent dye Fura-2 AM and pluronic were purchased from Molecular Probes (Eugene, OR). Dithiothreitol (sucinimidyl propionate) was obtained from Pierce. All other reagents were purchased from Sigma unless otherwise noted in the text.

**Cell Culture**—CHO-K1 sublines stably transfected with the M2 sub-type of human mACHR were maintained in complete medium consisting of Ham's F-12 medium (Cellgro Mediatech, Herndon, VA) containing 5% fetal bovine serum and 5 mM myo-2-glycerophosphate (Life Technologies, Inc.). After washing three times in lysis buffer, the immunoprecipitates were eluted with sample buffer (30 min, 4 °C), subjected to SDS-PAGE, and electrophotically transferred to PVDF membranes, and subjected to autoradiography.

**Immunoprecipitation of PLC-β with Gαq/11—**Immunoprecipitation of PLC-β1 or PLC-β3 with Gαq, has been described previously (26). CHO cells were incubated for 2 h in serum-free medium and exposed to agonists for 1 min in the presence of 1 mM dithiothreitol/sucinimidyl propionate to induce protein cross-linking and subsequently lysed in immunoprecipitation buffer described above. The lystate was centrifuged at 13,000 × g for 10 min, and the resulting supernatant was rotated (90 min, 4 °C) with Gαq/11 antibody and protein A-agarose beads (Life Technologies, Inc.). After washing three times in lysis buffer, the immunoprecipitates were eluted with sample buffer (30 min, 4 °C), subjected to SDS-PAGE, and electrophotically transferred to PVDF membranes. The PVDF membranes were blocked and probed by ECL-Western blotting as described below using antibodies to Gαq, PLC-β1, or PLC-β3.

**ECL-Western Blotting**—Cells were lysed by periodic agitation for 15 min in ice-cold lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, and 0.5% Nonidet P-40, pH 7.4) containing protease inhibitors (400 μM phenylmethylsulfonyl fluoride and 20 μg/ml leupeptin) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.2 mM sodium P2, and 10 mM β-glycerophosphate). After centrifugation at 16,000 × g for 10 min at 4 °C, the supernatants were diluted with lysis buffer to equal protein concentrations and boiled for 5 min with sample buffer (75 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue, pH 6.8). Aliquots containing equal protein concentrations were subjected to SDS-PAGE using a 5% stacking gel and a 10% separating gel and electrophotically transferred to PVDF membranes. The blocked PVDF membranes were incubated with antibodies to PLC-β1 or PLC-β3, followed by incubation with horseradish peroxidase-labeled anti-rabbit antibodies. Bound antibody was visualized by ECL and quantified by densitometry.

**Binding of [35S]GTPγS to Gαq/11—**Binding of [35S]GTPγS to Gαq/11 was measured by modification of a method described previously (26). CHO cells were washed in phosphate-buffered saline, suspended in reaction buffer (50 mM HEPES, 100 mM NaCl, 6 mM MgCl2, 2 mM EDTA, 10 μM GDP, 150 mM GTPγS, pH 7.4), and subjected to 70 °C freeze/thaw cycle to disrupt cell membranes. The freeze/thawed cells were incubated for 15 min (37 °C), with 30 nM [35S]GTPγS in the absence or presence of agonists for 10 min (37 °C). The samples were solubilized by rocking in lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM MgCl2, 100 mM GDP, 100 μM GTP, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 200 μg/ml leupeptin, pH 7.4). The samples were centrifuged (13,000 × g, 10 min, 4 °C), and the resulting supernatants were incubated (1.5 h, 4 °C) with Gαq/11 antibody and protein A-agarose. The immunoprecipitates bound to protein A-agarose were washed two times in lysis buffer, resuspended in distilled water, and transferred to scintillation vials containing Ultima-Gold scintillation fluid (Packard Bioscience). The amounts of [35S]GTPγS bound to the immunoprecipitated Gαq/11 were determined by liquid scintillation counting using an LS-6000 β-counter.

**Measurement of Gαq/11-stimulated PLC Activity in Membranes—**CHO cells were labeled for 24 h in Ham's F-12 medium containing 5% fetal bovine serum and 5 mM myo-2-glycerophosphate. The cells were lysed by periodic agitation for 15 min in phosphate-free Dulbecco's modified Eagle's medium containing 10 mM HEPES, pH 7.4, and 30 μM [32P]orthophosphoric acid.

**Immunoprecipitation of PLC-β1/β3—**Labeled cells were challenged with agonists for various times and subsequently lysed in immunoprecipitation buffer (50 mM HEPES, pH 7.4, 130 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 40 mM KH2PO4, 1% Triton X-100, 0.5% Nonidet P-40, 0.2% bovine serum albumin, 15% glycerol, 50 mM sodium fluoride, 1 mM sodium orthovanadate). The lystate was centrifuged at 13,000 × g for 10 min, and the resulting supernatant was rotated (90 min, 4 °C) with PLC-β1 or -β3 antibodies and protein A-agarose beads (Life Technologies, Inc.). After washing three times in lysis buffer, the immunoprecipitates were eluted with sample buffer (30 min, 4 °C), subjected to SDS-PAGE, and electrophotically transferred to PVDF membranes, and subjected to autoradiography.

**Co-immunoprecipitation of PLC-β with Gαq/11—**Co-immunoprecipitation of PLC-β1 or PLC-β3 with Gαq, has been described previously (26). CHO cells were incubated for 2 h in serum-free medium and exposed to agonists for 1 min in the presence of 1 mM dithiothreitol/sucinimidyl propionate to induce protein cross-linking and subsequently lysed in immunoprecipitation buffer described above. The lystate was centrifuged at 13,000 × g for 10 min, and the resulting supernatant was rotated (90 min, 4 °C) with Gαq/11 antibody and protein A-agarose beads.

**RESULTS**

To determine which PLC isoforms are expressed by CHO-m3 cells, PLC-β1 and -β3 were immunoprecipitated from [32S]-labeled CHO cells, submitted to SDS-PAGE, and visualized by...
autodigraphy (Fig. 1A). By this method we found that significant levels of PLC-β1 and -β3 are expressed by these cells. In contrast, neither PLC-β2 nor -β4 could be detected in immunoprecipitates or immunoblots of the CHO-m3 cells (Fig. 1B).

Previous studies indicate that GPCR activation can induce phosphorylation of PLC-β3 isoforms (17–19). We investigated this phenomenon by immunoprecipitating PLC-β1 and -β3 from 32P-labeled CHO-m3 cells exposed to carbachol or ATP. Both of these agonists induced a rapid and dose-dependent phosphorylation of PLC-β3 (Figs. 2 and 3) but not PLC-β1 (Fig. 3). Carbachol and ATP typically stimulated 4-fold and 3-fold increases in PLC-β3 phosphorylation above basal levels, respectively. These results indicate that PLC-β1 and -β3 are regulated differently by GPCR activation in these cells.

Confusion exists regarding the kinases mediating the GPCR-stimulated phosphorylation of PLC-β. Several studies have shown that both PKC and PKA can mediate this event, the latter being dependent on PKC activation (20). To clarify this controversy, we investigated whether PKC or PKA participates in PLC-β3 phosphorylation in CHO cells.

PKC is a ubiquitous serine/threonine kinase that is activated by diacylglycerol (21). It is known that this kinase can be activated by GPCR activation through the elevation of intracellular calcium levels and/or the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) (22). PKC is activated by GPCR stimulation through both these mechanisms. PKC, but not PKA, is activated by GPCR stimulation through the elevation of intracellular calcium levels, whereas PKA is activated by GPCR stimulation through the lowered intracellular calcium levels (23). PKC is activated by stimulation with GPCR agonists (21). It is known that PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms. PKC is activated by GPCR stimulation through both these mechanisms.

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indicated roles for both PKC (17, 18) and PKA (19). It is more likely that PKC, rather than PKA, acts in a classical negative feedback loop to mediate rapid PLC phosphorylation and deactivation. The involvement of PKA may indicate cross-regulation, which is unlikely to be a mechanism for rapid PLC phosphorylation and deactivation, because Gq11-coupled receptors do not generally activate adenyl cyclase. The ability of PKA to mediate agonist-induced phosphorylation of PLC-β3 was tested using the highly specific PKA antagonist, Rp-CAMPS. Concentrations of Rp-CAMPS well above those known to completely block PKA activation (28, 29) had no effect on the carbachol-induced phosphorylation of PLC-β3 at (Fig. 4). This finding indicates that PKA does not participate in PLC-β3 phosphorylation induced by the M₃ mAChR. The inability of PKA to mediate carbachol-induced PLC-β3 phosphorylation is not due to an inability of PKA to phosphorylate PLC-β3, because activation of PKA with forskolin induces PLC-β3 phosphorylation (Fig. 4).

In contrast to the effects of inactivating PKA, inactivation of PKC with the specific antagonist BIM significantly inhibited the carbachol- or ATP-induced phosphorylation of PLC-β3 (Fig. 4A). This finding indicates that PKC participates in the agonist-induced phosphorylation of PLC-β3. Further evidence that PKC mediates PLC-β3 phosphorylation is provided by our finding that PLC-β3 is phosphorylated when PKC is directly activated by PMA (Figs. 3 and 4). PMA typically increased PLC-β3 phosphorylation 3–4-fold above basal levels. Interestingly, activation of PKC does not induce the phosphorylation of PLC-β1 (Fig. 4B). This finding suggests that the lack of agonist-induced phosphorylation of PLC-β1 is due to an inability of PLC-β1 to serve as a substrate for PKC-mediated phosphorylation in these cells.

The mechanism by which PKC inhibits PLC-β is poorly characterized. We found that treatment with PMA diminishes GTPγS-stimulated inositol polyphosphate generation in the CHO-m3 cells (Fig. 5). This result indicates that PKC stimulation diminishes the ability of GTPγS to activate PLC-β. This event may occur because PKC stimulation diminishes the ability of activated Gq11 proteins to associate with PLC-β1. To investigate this possibility, we determined whether PKC stimulation alters the co-precipitation of PLC-β with Gq11 in agonist-treated cells (Fig. 6). Carbachol induces Gq11 to associate with PLC-β1 (Fig. 6A, lane 3) and PLC-β3 (Fig. 6B, lane 3). Treatment with PMA completely abolishes the carbachol-induced association of Gq11 with PLC-β1 (Fig. 6B, lane 6). This result suggests that the PKC-mediated phosphorylation of PLC-β3 diminishes the interaction of Gq11 with the phospholipase. This response is not restricted to signaling by the muscarinic receptor, because treatment with PMA also diminishes the ATP-induced association of Gq11 with PLC-β3 (Fig. 6B, lane 5). Treatment with PMA does not alter the agonist-induced association of Gq11 with PLC-β1 (Fig. 6A, lane 6), consistent with our finding that PMA has no effect on PLC-β1 phosphorylation.
To determine whether PKC activation alters other aspects of receptor signaling, the effects of PMA on the agonist-induced activation of Goq/11 were investigated. Activation of P2Y2 receptors stimulates an approximately 500% increase in Goq/11 activation, as indicated by the binding of [35S]GTPγS to Goq/11 (Fig. 7A). This response is unaffected by pretreatment with PMA (Fig. 7A). Similarly, the M3 mACHR-stimulated increase in Goq/11 activation is unaffected by PMA pretreatment (Fig. 7A). These findings indicate that the GPCR-dependent activation of Goq/11 is intact in PMA-treated cells. Consistent with this result, we found that PMA treatment actually enhances the ability of the muscarinic receptor to activate phospholipase A2, as indicated by arachidonic acid release (Fig. 7B).

The preceding results indicate that the GPCR-mediated activation of PKC stimulates PLC-β3 phosphorylation, resulting in reduced PLC-β3 activity because of diminished interactions of the phosphorylated PLC-β3 with Goq/11. This model predicts that the activation of PLC-β3 by GPCR should be enhanced when PKC activity is inhibited and diminished when PKC activity is stimulated. To test this prediction, we measured the effects of pharmacologically altering PKC activity on GPCR-mediated Ca2+ mobilization, which is an indicator of PLC-β activity. We found that inactivation of PKC with BIM enhances Ca2+ mobilization induced by ATP (Fig. 8A) and carbachol (Fig. 8B). Conversely, activation of PKC with PMA diminishes Ca2+ mobilization induced by these agonists (Fig. 8). These findings support the model that the PKC-mediated phosphorylation of PLC-β3 diminishes the activity of the phospholipase.

**DISCUSSION**

The purpose of this investigation was to characterize the involvement of PLC-β phosphorylation in the termination of GPCR-dependent PLC activation. Activation of PLC-β by P2Y2 receptors and M3 mACHR was investigated because these GPCRs are known to stimulate transient and sustained PLC-β activity, respectively. Our results indicate that activation of these receptors induces the PKC-mediated phosphorylation of PLC-β3. This phosphorylation event may deplete PLC-β3 by inhibiting the association of Goq/11 with the phospholipase. In contrast, PLC-β1 is not phosphorylated by PKC and thus is not deactivated by this mechanism. Our results suggest that the activation of PLC-β1 by M3 mACHR, but not by P2Y2 receptors, may contribute to the sustained PLC-β2 activity induced by M3 mACHR activation. This model is depicted in Fig. 9.

The activation of PLC-β by GPCRs typically occurs and declines rapidly (6–8). The GPCR-induced activation of PLC-β is usually detectable in just a few seconds and frequently peaks around 10–20 s before falling either to the base line or to a plateau level above the base line. These changes in PLC-β activity are mirrored by similar changes in [Ca2+]i concentration. Negative feedback inhibition of PLC-β by PKC is one mechanism that has been proposed for the rapid termination of phosphatidylinositol 4,5-bisphosphate hydrolysis and [Ca2+]i mobilization (4, 17, 18). However, investigations addressing the effects of PKC activation on the GPCR-activated PLC-β cascade have been limited by the inability to conclusively pinpoint the site of PKC action.

Because of the large number and complexity of components involved, PKC could act at many sites in the GPCR signaling pathway to limit PLC-β activity. Direct activation of PKC with PMA is known to induce the phosphorylation of M3 mACHR, with undetermined effects on M3 mACHR function (12). This finding, along with demonstrations that some other GPCRs can be phosphorylated by PKC (22, 23), led to the speculation that PKC activation inhibits events at the receptor level. However, we found that activation of PKC with PMA does not alter the ability of M3 mACHR or P2Y2 receptors to activate Goq/11. This result indicates that PKC inhibits a post-receptor event. Consistent with this conclusion, we found that PKC activation inhibits IP3 generation that is induced by directly activating G proteins with GTPγS, without involving GPCRs. These findings indicate that PKC inhibits the GPCR signaling cascade only at a point after the activation of Goq/11. Our studies show that PKC activation induces the phosphorylation of PLC-β3 and diminishes the ability of PLC-β3 to interact with Goq/11. In contrast, PKC activation does not induce the phosphorylation of PLC-β1, nor does it affect the ability of PLC-β1 to associate with Goq/11. These findings suggest that PKC inhibits PLC-β3 activity by phosphorylating PLC-β3 and diminishing the ability of PLC-β3 to interact with Goq/11. Although PKC activation may inhibit other events following PLC-β activation, our results indicate that the PKC-mediated PLC-β Phosphorylation 39771

**FIG. 8.** PKC modulates Ca2+ mobilization induced by M3 mACHR or P2Y2 receptor activation. Ca2+ mobilization induced by 10 μM ATP (A) or 0.5 μM carbachol (B) was measured using CHO cells that had been preincubated for 10 min with 10 μM BMW, 50 nM PMA, or no drug. Results are representative of three independent experiments that produced similar results.

**FIG. 9.** A model depicting how PKC may differentially modulate PLC-β activity induced by M3 mACHR or P2Y2 receptor activation. M3 mACHR activate both PLC-β1 and-β3 and a greater proportion of Goq/11 than P2Y2 receptors. P2Y2 receptors activate PLC-β3 but show no detectable activation of PLC-β1. Activation of PLC-β3 but not PLC-β1 is subject to PKC-mediated negative feedback inhibition, resulting in rapid termination of receptor-activated PLC-β3 activity. Phosphorylation of PLC-β3 results in a decreased association of Goq/11 with PLC-β3. Selective activation of PLC-β1 or -β3, together with selective PKC-mediated negative feedback inhibition, provide at least a partial explanation for the mechanisms allowing prolonged versus transient receptor-mediated activation of PLC-β.
mediated inhibition of Goq11 and PLC-β interactions is one way that the GPCR signaling cascade can be deactivated.

It is intriguing that PKC activation induces phosphorylation of PLC-β3 but not PLC-β1. The reason for the lack of PLC-β1 phosphorylation is unknown. It is unlikely that any differences in the intracellular compartmentalization of PLC-β1 and PLC-β3 contribute to their different phosphorylation profiles in CHO-m3 cells, because PMA probably has equal access to different intracellular sites. It is conceivable that PLC-β1, but not PLC-β3, is associated intracellularly with protein partners that protect it from PKC-mediated phosphorylation, although evidence for this possibility is lacking.

Activation of PKA, rather than PKC, has been found to induce PLC-β3 phosphorylation in some systems (19). This PKA-mediated phosphorylation of PLC-β3 would represent cross-regulation between GPCRs that signal through adenylyl cyclase- and PLC-β-mediated pathways and not negative feedback control. This distinction is critical because PKC-mediated negative feedback can be a mechanism for the termination of PLC activity. In contrast, PKA-mediated cross-regulation is unlikely to be a mechanism for the termination of PLC activity because Goq11-coupled GPCRs do not generally activate adenylyl cyclase. We demonstrated that PLC-β3 phosphorylation induced by P2Y2 receptors or M3 mAChR is not mediated by the action of PKA nor by an increase in cAMP. Agents that elevate cAMP do not affect PLC-β activation by these receptors. Similarly, it was previously reported that signaling by the platelet-activating factor receptor, which activates PLC-β via Goq11 and induces PLC-β3 phosphorylation, is insensitive to the action of forskolin or other PKA-activating agents (30). However, we did find that agents that increase cAMP can stimulate modest increases in PLC-β3 phosphorylation (typically 1.5-fold). It is possible that PKA regulates PLC-β activity only when PLC-β is activated by specific GPCR-mediated signals that differ from those generated by M3 mAChR or P2Y2 receptor stimulation in CHO cells. This possibility is supported by a previous report that PKA activation alters signaling by the formyl peptide receptor (30), which activates PLC-β via Goq11 rather than via Goq11.

The differential coupling of M3 mAChR and P2Y2 receptors to PLC-β1 and PLC-β3 was somewhat surprising. Whereas the stimulation of M3 mAChR induced the interaction of Goq11 with PLC-β1 and PLC-β3, the stimulation of P2Y2 receptors only induced the interaction of Goq11 with PLC-β3. These differences may occur because greater levels of Goq11 are activated by stimulating M3mAChR than by stimulating P2Y2 receptors in these cells. Stimulation of P2Y2 receptors may not activate enough Goq11 to allow the detectable association of PLC-β1 with Goq11 in our assays. Alternatively, it is possible that PLC-β1 is located in unique intracellular sites that make it more susceptible to activation by M3 mAChR than by P2Y2 receptors.

Our results suggest an explanation for the ability of M3 mAChR to induce sustained PLC-β activation. Sustained PLC-β activity is induced by stimulating endogenous M3 mAChR in cultured neuronal and lung cancer cell lines (6, 9), indicating that sustained activation of PLC-β is not simply a result of nonphysiological signaling by transfected mAChR.

Instead, our results suggest that sustained PLC-β activation may be induced by M3 mAChR because these receptors activate PLC-β1, which is not susceptible to PKC-dependent inhibition. In contrast, only transient PLC-β activation may be induced by P2Y2 receptors because these receptors activate only PLC-β3, which is susceptible to PKC-mediated inhibition. We found that inactivation of PKC with BIM converts P2Y2 receptor-mediated [Ca2+]i mobilization from a transient event to a sustained event. According to our model, treatment with BIM diminishes the PKC-mediated inhibition of PLC-β3, resulting in prolonged activation of PLC-β3 and sustained [Ca2+]i mobilization after P2Y2 receptor activation. In addition to this PKC-mediated regulation of PLC-β, other negative and positive feedback events undoubtedly contribute to the different abilities of the M3 mAChR and P2Y2 receptor to regulate PLC-β activity. However, the activation of specific PLC-β isoforms that have different susceptibilities to PKC-mediated inhibition provides an intriguing mechanism for the temporal regulation of PLC-β activity by GPCRs.

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