Stimulation of Phospholipase C-ε by the M₃ Muscarinic Acetylcholine Receptor Mediated by Cyclic AMP and the GTPase Rap2B*

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Stimulation of phospholipase C (PLC) by Gₐ-coupled receptors such as the M₃ muscarinic acetylcholine receptor (mAChR) is caused by direct activation of PLC-β enzymes by Gαₐ proteins. We have recently shown that G₂-coupled receptors can stimulate PLC-ε, apparently via formation of cyclic AMP and activation of the Ras-related GTPase Rap2B. Here we report that PLC stimulation by the M₃ mAChR expressed in HEK-293 cells also involves, in part, similar mechanisms. M₃ mAChR-mediated PLC stimulation and [Ca²⁺], increase were reduced by 2',5'-dideoxyadenosine (dd-Ado), a direct adenyl cyclase inhibitor. On the other hand, overexpression of Gαₐ or Epac1, a cyclic AMP-regulated guanine nucleotide exchange factor for Rap GTPases, enhanced M₃ mAChR-mediated PLC stimulation. Inactivation of Ras-related GTPases with clostridial toxins suppressed the M₃ mAChR responses. The inhibitory toxin effects were mimicked by expression of inactive Rap2B, but not of other inactive GTPases (Rac1, Ras, RalA, Rap1A, and Rap2A). Activation of the M₃ mAChR induced GTP loading of Rap2B, an effect strongly enhanced by overexpression of Gαₐ and inhibited by dd-Ado. Overexpression of PLC-ε and PLC-β₁, but not PLC-β₁ or PLC-δ₁, enhanced M₃ mAChR-mediated PLC stimulation and [Ca²⁺], increase. In contrast, expression of a catalytically inactive PLC-ε mutant reduced PLC stimulation by the M₃ mAChR and abrogated the potentiating effect of Gαₐ. In conclusion, our findings suggest that PLC stimulation by the M₃ mAChR is a composite action of PLC-β₁ stimulation by Gαₐ and stimulation of PLC-ε apparently mediated by G₂-dependent cyclic AMP formation and subsequent activation of Rap2B.

Stimulation of phosphatidylinositol 4,5-bisphosphate (PIP₂)₁-hydrolyzing phospholipase C (PLC) is a major signal transduc-

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‡ The abbreviations used are: PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; GPCR, G protein-coupled receptor; GEF, guanine nucleotide exchange factor; PTX, pertussis toxin; mAChR, muscarinic acetylcholine receptor; IP₂, inositol 1,4,5-trisphosphate; dd-Ado, 2',5'-dideoxyadenosine; RapGDS-RBD, Rap-binding domain of the Ras guanine nucleotide dissociation stimulator; RGS, regulator of G protein signaling; HA, hemagglutinin; GTPγS, guanosine 5'-O-(thiotriphosphate).

Materials—myo-[³²H]inositol (10–25 Ci/mmol) and d-myo-[³²H]inositol 1,4,5-trisphosphate ([³²H]IP₃; 21 Ci/mmol) were from PerkinElmer Life Sciences. Unlabeled IP₃ was from Biomol, 2',5'-dideoxyadenosine (dd-Ado) was from Calbiochem-Novabiochem, and Fura-2/AM was from Molecular Probes. The antibodies against Rac1, Rap1A, Rap2, PLC-β₁, PLC-γ₁, and PLC-δ₁ were from Santa Cruz. The antibodies against RalA and Ras were from Transduction Laboratories. The antibody against HA-tagged proteins (12CA5) was a kind gift of Dr. J. L. Bos. The polyclonal rabbit anti-PLC-ε antibody raised against the unique N-
terminal 600 amino acids of PLC-ε did not recognize other PLC isoforms (not shown). *Clostridium difficile* toxin B-1470 and *Clostridium sordellii* lethal toxin (strains 82 and 1522, respectively) were kind gifts of Dr. C. von Eichel-Streiber.

**Expression Plasmids and Transfection**—cDNAs encoding the inactive GTPase mutants of Ras1 (T17N Ras1; subcloned into pEXV), RaLa (G26A RaLa), Rap1A (S17N Rap1A), Rap2A (S17N Rap2A), and Rap2B (S17N Rap2B; each subcloned into pRK5) were kindly provided by Drs. D. Illenberger and A. Ullrich. cDNAs encoding the constitutively active mutant of Goq12 (Q229L Goq12; subcloned into pCis), FLAG-tagged RGS4 (subcloned into pCMV), and wild-type HA-tagged Goq (subcloned into pcDNA3) were kindly provided by Drs. T. Wieland and C. Kleuss. cDNAs encoding PLC-β1 and PLC-γ1 (both subcloned into pRK5) were kindly provided by Drs. D. Illenberger and A. Ullrich. PLC-ε, wild-type PLC-ε, and catalytically inactive PLC-ε (H1144L PLC-ε) were each subcloned into pcDNA3. HEK-293 cells stably expressing M₃ mAChR at high density (11) were cultured as reported previously (14). Transfection of cells grown to near confluence on 145-mm culture dishes with the indicated amounts of either plasmid DNA or the corresponding empty vectors was performed with the calcium phosphate method, reaching a transfection efficiency of 50–60% (14). Expression of the encoded proteins was verified by the immunoblotting of cell lysates with specific antibodies. Assays were performed 48 h after transfection.

**Measurement of PLC Activity**—For measurement of inositol phosphate formation, cellular phospholipids were labeled by incubating cells for 24 h with myo-[³²P]inositol (0.5 μCi/ml) in growth medium. Thereafter, the adherent cells were first treated for 10 min at 37 °C in Hanks’ balanced salt solution, containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM d-glucose, buffered at pH 7.4 with 15 mM HEPES, plus 10 mM LiCl, followed then by further incubation for 30 min at 37 °C in the presence of the agent or its solvent, dimethyl sulfoxide (0.1%); dd-Ado was also present during the PLC assays. To study the effects of dd-Ado, the cells were pretreated for 30 min with the agent or its solvent, dimethyl sulfoxide (0.1%); dd-Ado was also present during the PLC assays. To study the effects of Clostridial toxins, the cells were treated for 24 h without and with the toxins at the indicated concentrations, followed by PLC activity assays.

**Calcium Measurements**—Intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined in cell suspensions with the fluorescent Ca²⁺ indicator dye Fura-2 in a Hitachi spectrofluorometer as described previously (17).

**Activation of Rap2B**—Cells were stimulated without and with carbachol for 5 min at 37 °C, followed by two washes with ice-cold phosphate-buffered saline and lysis in a buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 μg/ml PMSF, 5 mM MgCl₂, and 2 mM CaCl₂, plus 10 mM LiCl and 0.1% dimethyl sulfoxide: 100 μg/ml of the catalytically inactive Gε12 mutant of Gε12 (H1144L Gε12) was added to the cultures, and the concentration of Gε12 was increased in steps of 100 μg/ml from a final concentration of 1 μg/ml up to 100 ng/ml as described by others (17).
trypsin inhibitor, 10 mM NaF, and 1 mM Na$_3$VO$_4$. After centrifugation, the supernatants were incubated with 15 µg of purified glutathione S-transferase-tagged Rap-binding domain of the Ral guanine nucleotide dissociation stimulator bound to glutathione-Sepharose beads for 1 h at 4°C. Then, the beads were washed three times with lysis buffer and finally incubated in Laemmli buffer for 10 min at 95°C. Bound Rap2-GTP was determined by immunoblotting with an anti-Rap2 antibody (15, 18). Densitometric analysis of the bands was performed with ImageQuant software (Molecular Dynamics).

**RESULTS**

**Role of Cyclic AMP in $M_3$ mAChR-mediated PLC Stimulation**—We have recently reported that stimulation of cyclic AMP formation by the $\beta_2$-adrenoreceptor expressed in HEK-293 cells or direct activation of adenylyl cyclase by forskolin results in PLC stimulation (15). To examine whether cyclic AMP and cyclic AMP-dependent processes are involved in PLC stimulation by the $M_3$ mAChR as well, several approaches were used. First, we studied the effect of the P-site adenylyl cyclase inhibitor, dd-Ado (19), on PLC stimulation by the $M_3$ mAChR stably expressed in HEK-293 cells. Treatment of the cells with dd-Ado (100 µM) had no effect on unstimulated inositol phosphate accumulation or IP$_3$ levels. However, dd-Ado strongly reduced, by 30–40%, IP$_3$ formation or inositol phosphate accumulation (see below) stimulated by the mAChR agonist, carbachol (Fig. 1A). In line with this inhibition, dd-Ado significantly ($p < 0.0001$) reduced the carbachol (1 µM)-induced increase in [Ca$^{2+}$]$_i$, from 485 ± 25 nM to 246 ± 35 nM ($n = 8–10$). Second, we examined whether overexpression of G$_{\alpha_s}$, the adenylyl cyclase-stimulatory G protein, alters PLC stimulation by the $M_3$ mAChR. In cells overexpressing G$_{\alpha_s}$, basal inositol phosphate formation...
was not altered. However, PLC stimulation induced by carbachol (1 μM) was increased by about 50%. This increase in PLC stimulation was almost fully suppressed by treatment of the cells with 10 μM dd-Ado (Fig. 1B). Together, these data suggested that PLC stimulation by the M₃ mAChR is, at least in part, dependent on cyclic AMP formation.

As observed with β₂-adrenoreceptor-induced PLC stimulation (15), treatment of HEK-293 cells with the protein kinase A inhibitor, H-89 (10 μM), had no significant effect on PLC stimulation induced by carbachol, neither in control cells nor in cells overexpressing Gαs (data not shown), suggesting that the action of cyclic AMP is mediated by another effector. Therefore, we overexpressed Epac1, a cyclic AMP-activated GEF for Rap GTPases (18, 20). As illustrated in Fig. 2A, similar to Gαs, overexpression of Epac1 had no effect on basal PLC activity, but strongly enhanced PLC stimulation induced by carbachol (1 μM). Overexpression of Epac1 also strongly enhanced the potency of carbachol to increase [Ca²⁺]ᵢ in HEK-293 cells. While carbachol increased [Ca²⁺]ᵢ in control cells with an EC₅₀ value of 450 ± 14 nM, this value was reduced by about one order of magnitude, to 26 ± 2 nM, in cells overexpressing Epac1 (Fig. 2A). These data suggested that cyclic AMP/Epac1-controlled Rap GTPases are involved in PLC stimulation by the M₃ mAChR.

FIG. 3. Effects of various inactive GTPase mutants on M₃ mAChR-induced PLC and calcium signaling. HEK-293 cells were transfected with empty vector (Control, V), T17N Rac1, S17N Ras, G26A RalA, S17N Rap1A, S17N Rap2A, or S17N Rap2B (100 μg of DNA each). At 48 h after transfection, [³H]inositol phosphate formation without (Basal) and with 1 μM carbachol (A) or [Ca²⁺]ᵢ increase induced by 1 μM carbachol was determined (B). Insets, immunoblot detection of the GTPases in lysates of transfected cells. Data in A are means ± S.E. (n = 5), while in B superimposed tracings of [Ca²⁺]ᵢ are shown.
Involvement of Rap2B in M₃ mACHR-mediated PLC Stimulation—To study whether and which type of Rap GTPases are involved in PLC stimulation by the M₃ mACHR, we first examined the effects of C. difficile toxin B-1470 and C. sordellii lethal toxin, known to inactivate Ras-related GTPases (21, 22). Treatment of HEK-293 cells for 24 h with 300 pg/ml toxin B-1470 and 100 ng/ml lethal toxin strongly reduced inositol phosphate accumulation (data not shown) and IP₃ formation induced by carbachol (Fig. 2B). PLC stimulation induced by the direct G protein activators, AlF₄⁻ (intact cells) and GTPγS (permeabilized cells), was also strongly reduced in cells treated with the toxins (data not shown). The reduction in M₃ mACHR-mediated IP₃ formation was paralleled by an attenuation of receptor-mediated [Ca²⁺]ᵢ increase. While carbachol (1 μM) increased [Ca²⁺]ᵢ by 485 ± 50 nM (n = 8) in control cells, this increase was significantly (p < 0.001) reduced to 305 ± 35 nM (n = 10) and 265 ± 25 nM (n = 6) in cells treated with toxin B-1470 and lethal toxin, respectively (Fig. 2B). Treatment of the cells with the toxins, however, did not alter the number of cell surface M₃ mACHRs, determined by binding of the membrane-impermeant mACHR antagonist, N-[³²P]methylscopolamine, in intact cells (16), and the carbachol-induced binding of [³⁵S]Giuinosine 5’-O-(3-thiotriphosphate) to G proteins, measured in permeabilized cells (23). Furthermore, the toxins had no effect on the cellular IP₃ levels, measured as [³H]IP₃ or [³H]IP₃ mass in control and toxin-treated cells (24), and did not reduce Ca²⁺ (1 μM)-stimulated PLC activity, measured with exogenous IP₃ in cell lysates (17) (data not shown). Thus, inhibition of PLC and calcium signaling by the GTPase-inactivating toxins was apparently not due to a loss of cell surface receptors, a defective receptor-G protein coupling, a fall in PLC substrate levels, or a general reduction in PLC activities, suggesting that inhibition of M₃ mACHR signaling to PLC by the toxins is caused by inactivation of small GTPases specifically involved in PLC stimulation.

To identify the specific GTPase, we expressed inactive mutants of the GTPases serving as toxin substrates. Compared with the expression of the endogenous GTPases, the various GTPase mutants were overexpressed to a comparable level (Fig. 3A). However, the GTPase mutants largely differed in their effects on PLC signaling. Expression of inactive Rac1, H-Ras, RaLa, RaLB (not shown), Rap1A, and Rap2A did neither change basal PLC activity nor PLC stimulation by the M₃ mACHR. In contrast, in cells expressing S17N Rap2B PLC stimulation induced by carbachol was reduced by 40–50% (Fig. 3A). In line with their distinct effects on PLC stimulation, expression of S17N Rap2B, but not S17N Ras or S17N Rap1A (not shown), strongly (p < 0.0001) reduced the carbachol (1 μM)-induced [Ca²⁺]ᵢ increase, from 535 ± 55 nM in control cells to 310 ± 45 nM (n = 6–8) in cells expressing S17N Rap2B (Fig. 3B).

Next, we studied whether Rap2B is activated by the M₃ mACHR and whether this activation is affected by agents inhibiting or enhancing receptor-mediated PLC stimulation. Carbachol (1 μM) treatment of HEK-293 cells enhanced GTP loading of endogenous and overexpressed Rap2B, as determined by extraction of the GTPase from cell lysates with immobilized RalGDS-RBD (Fig. 4). Expression of constitutively active Gα₁₂ (Q229L Gα₁₂) or overexpression of wild-type Gα₁₂ (not shown), both of which did not alter carbachol-induced PLC stimulation (14, not shown), had no effect on M₃ mACHR-induced Rap2B activation. In contrast, overexpression of Gαᵦ or Epac1 (not shown), which by themselves did not alter the activity state of Rap2B, strongly enhanced the stimulatory effect of carbachol (Fig. 4). Treatment of the cells with dd-Ado (10 μM) almost completely (by 90 ± 5%; n = 4) abrogated the potentiating effect of Gαᵦ (data not shown). Thus, the M₃ mACHR activates Rap2B and, similarly as observed for PLC stimulation, this activation is apparently controlled by cyclic AMP.

Stimulation of PLC-ε by the M₃ mACHR—We next sought to determine which PLC isozyme is activated by the M₃ mACHR. For this, we examined the effects of overexpression of PLC-β₁, PLC-γ₁, PLC-δ₁, and PLC-ε on basal and receptor-stimulated PLC activities. As illustrated in Fig. 5A, overexpression of PLC-γ₁, which increased PLC stimulation by epidermal growth factor (not shown), did neither change basal PLC activity nor its stimulation by carbachol (1 μM). In cells overexpressing PLC-δ₁, basal PLC activity was increased by 2.5-fold, whereas carbachol-stimulated PLC activity was not altered. In contrast, overexpression of PLC-β₁ and PLC-ε enhanced PLC stimulation by carbachol by 50 and 70%, respectively, without altering basal PLC activity. In line with these data, carbachol (1 μM)-induced [Ca²⁺]ᵢ increase was strongly potentiated in cells overexpressing PLC-β₁ or PLC-ε from 470 ± 55 nM in control cells to 700 ± 45 nM in cells overexpressing PLC-β₁ (n = 6–8; p < 0.0001) and from 524 ± 35 nM to 940 ± 65 nM in cells overexpressing PLC-ε (n = 8–10; p < 0.0001) (Fig. 5B).
In contrast to wild-type PLC-ε, expression of the catalytically inactive PLC-ε mutant, H1144L PLC-ε/H9280, reduced PLC stimulation by carbachol by 25% (Fig. 6A). Interestingly, expression of H1144L PLC-ε/H9280 almost fully reversed the potentiating effect of co-expressed Gq/H9251s, suggesting that potentiation of PLC stimulation by Gq/H9251s is due to activation of the PLC-ε isozyme. This assumption was corroborated by studies with the adenylyl cyclase inhibitor, dd-Ado, in cells overexpressing PLC-ε/H9280 or PLC-ε/H9252. As shown in Fig. 6B, dd-Ado (10 μM) strongly reduced the potentiating effect of overexpressed PLC-ε on carbachol-stimulated inositol phosphate formation, whereas dd-Ado was without effect in cells overexpressing PLC-β1, suggesting that cyclic AMP is involved in stimulation of PLC-ε, but not PLC-β1, by the M₃ mAChR.

We have recently reported that overexpression of the regulator of G protein signaling 4 (RGS4), which acts as a GTPase-activating protein for Gq and Gs proteins (25, 26), strongly reduces M₃ mAChR-mediated PLC stimulation was reduced by about 40%. Most important, the inhibitory effect of dd-Ado (10 μM) on PLC stimulation was fully retained in cells overexpressing RGS4. In contrast, in cells expressing S17N Rap2B, in which the M₃ mAChR response was reduced to a similar extent as in cells overexpressing RGS4, treatment with dd-Ado did not cause a further reduction in receptor-mediated PLC stimulation (Fig. 7B).

**DISCUSSION**

PTX-insensitive stimulation of PLC by GPCRs is generally assumed to be caused by direct activation of PLC-β isozymes by activated α-subunits of Gq type G proteins (3, 4). The M₃ mAChR is a prototypical example of such GPCRs. PLC stimulation by the M₃ mAChR that preferentially couples to Gq proteins of the Gq family is PTX-insensitive (11–13). Furthermore, we have recently reported that PLC stimulation by the M₃ mAChR is specifically suppressed by RGS proteins inactivating PTX-resistant Gqα₄, but not Gqα₁₁ type G proteins (14). Interestingly, overexpression of the Gqα₄-inactivating RGS4 reduced
PLC stimulation by the M₃ mAChR only partially, by 60–70\% (14). In studies with Gₛ-coupled receptors, we made very recently the unexpected observation that such receptors, i.e. the β₂-adrenoreceptor expressed in HEK-293 cells and the prostanoid receptor endogenously expressed in N1E-115 neuroblastoma cells, can also mediate PLC stimulation (15). This PLC stimulation was PTX-insensitive and apparently mediated by Gₛ-dependent formation of cyclic AMP and activation of the Ras-related GTPase Rap2B, finally resulting in stimulation of the PLC-₁ isozyme (15). As the M₃ mAChR can couple to Gₛ and increase cyclic AMP formation (11, 27), we examined in the present study whether PLC stimulation by the M₃ mAChR may involve similar mechanisms. We report here that PLC stimulation by the M₃ mAChR expressed in HEK-293 cells is a composite action on PLC-₁ and PLC-ᵢ isozymes and that stimulation of PLC-ᵢ by the M₃ mAChR is apparently mediated by Gₛ-dependent cyclic AMP formation and activation of the GTPase Rap2B.

First, treatment of the cells with the P-site adenylyl cyclase inhibitor, dd-Ado, reduced PLC stimulation by the M₃ mAChR. Second, overexpression of Gₛ, which by itself had no effect on PLC activity, strongly enhanced M₃ mAChR signaling to PLC. Third, a similar enhancement of M₃ mAChR-mediated PLC stimulation was observed in cells overexpressing the cyclic AMP-activated GEF for Rap GTPases, Epac1, while inhibition of cyclic AMP-dependent protein kinase A by H-89 was without effect. Fourth, inactivation of Ras-related GTPases with C. difficile toxin B-1470 and C. sordellii lethal toxin strongly reduced PLC stimulation. Fifth, the inhibitory toxin effects were mimicked by expression of an inactive Rap2B mutant, but not by inactive mutants of other GTPases serving as toxin substrates. Sixth, the M₃ mAChR induced activation of Rap2B, and this activation was enhanced by overexpression of Gₛ or Epac1 and suppressed by dd-Ado. Seventh, PLC stimulation by the M₃ mAChR was enhanced by overexpression of PLC-ᵢ, similar to overexpression of PLC-₁, and reduced by expression of a catalytically inactive PLC-ᵢ mutant. Finally, using various combinations, i.e. Gₛ with H1144L PLC-ᵢ and dd-Ado with PLC-₁, PLC-ᵢ, RGS4, and S17N Rap2B, evidence is provided that cyclic AMP-dependent PLC stimulation by the M₃ mAChR involves Rap2B and the PLC-ᵢ isozyme and that this stimulation is largely independent of PLC-₁ stimulation by Gₛ.

Activation of Gₛ and stimulation of adenylyl cyclase is not considered a primary function of the M₃ mAChR, compared with coupling to Gᵢ and stimulation PLC-ᵢ isozymes (12). Therefore, we were surprised to observe that inhibition of adenylyl cyclase by dd-Ado and overexpression of cyclic AMP-activated Epac1 had such marked effects on M₃ mAChR-mediated PLC stimulation. Similarly as reported before by others (11), increases in total cellular cyclic AMP levels in HEK-293
cells expressing the M₃ mAChR were observed only at rather high carbachol concentrations (>1 μM) (data not shown). However, as shown herein, inhibition of M₃ mAChR-mediated PLC stimulation by dd-Ado was largely independent of the carbachol concentration used (Fig. 1A), and overexpression of Epac1 markedly increased PLC stimulation and [Ca²⁺] levels via stimulation of PLC-ε isozymes, activates Rap2B and thereby PLC-ε. Such a reaction may also explain why overexpression of PLC-ε enhanced M₃ mAChR-mediated PLC stimulation at least as efficiently as overexpression of PLC-β1 (see Fig. 5). The involvement of additional Rap-GEFs in the M₃ mAChR response will be addressed in future studies.

In conclusion, we report here that PLC and calcium signaling by the M₃ mAChR is mediated by the two PTX-insensitive G proteins, Gₛ and Gₓ, resulting in stimulation of PLC-β1 and PLC-ε, respectively. While stimulation of PLC-β1 is most likely caused by direct interaction with activated Goₓ proteins, stimulation of PLC-ε is apparently dependent on cyclic AMP formation and activation of Epac1 and in consequence Rap2B. This GTase seems also to be involved in overall PLC stimulation at least as efficiently as overexpression of PLC-β1 (see Fig. 5). The involvement of additional Rap-GEFs in the M₃ mAChR response will be addressed in future studies.

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