ADP-ribosylation Factor-dependent Phospholipase D Activation by the M₃ Muscarinic Receptor*

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G protein-coupled receptors can potentially activate phospholipase D (PLD) by a number of routes. We show here that the native M₃ muscarinic receptor in 1321N1 cells and an epitope-tagged M₃ receptor expressed in COS7 cells substantially utilize an ADP-ribosylation factor (ARF)-dependent route of PLD activation. This pathway is activated at the plasma membrane but appears to be largely independent of Gq/11, phospholipase C, Ca²⁺, and protein kinase C, tyrosine kinases, and phosphatidyl inositol 3-kinase. We report instead that it involves physical association of ARF with the M₃ receptor as demonstrated by co-immunoprecipitation and by in vitro interaction with a glutathione S-transferase fusion protein of the receptor’s third intracellular loop domain. Experiments with mutant constructs of ARF1/6 and PLD1/2 indicate that the M₃ receptor displays a major ARF1-dependent route of PLD1 activation with an additional ARF6-dependent pathway to PLD1 or PLD2. Examples of other G protein-coupled receptors assessed in comparison display alternative pathways of protein kinase C- or ARF6-dependent activation of PLD2.

Many G protein-coupled receptors (GPCRs)¹ can activate phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatic acid and choline. Both phosphatidates and diacylglycerols (formed by phosphatidate hydrolysis) may act as intracellular messengers. PLD has been implicated as a key regulator of vesicular trafficking, cytoskeletal organization, exocytosis, endocytosis, and further signaling pathways (1–4). Activation of PLD can be brought about by a variety of signaling events (5–8), many of which could potentially contribute to the stimulation of PLD activity by GPCRs. These include the activation of protein kinase C (PKC), protein-tyrosine kinases, phosphatidylinositol 3-kinase (PI 3-kinase), small G proteins of the ARF and Rho families, and possibly the elevation of intracellular Ca²⁺ levels.

This study addresses the mechanism of PLD activation by the M₃ muscarinic receptor expressed endogenously in 1321N1 human astrocytoma cells and heterologously in COS7 cells. The M₃ receptor is a member of the Group I, rhodopsin-related GPCR family that is expressed in the nervous system and peripheral tissues. The best established signaling pathway from the M₃ receptor is the pertussis toxin-insensitive activation of phospholipase C (PLC) via the heterotrimeric G protein Gq/11, although PLD is also strongly activated. In various cell types, PKC, protein-tyrosine kinases, ARF, and Rho have each been specifically implicated in M₃ receptor-mediated PLD activation (6, 9–12). The data here emphasize the importance of a pathway to PLD that involves direct association between ARF and the M₃ receptor (12).

ARF1 and ARF6 are representative of the main classes of cellular ARFs (Classes I and III) and have distinct subcellular distributions in many cell types. In resting cells, ARF1 is largely cytosolic or Golgi-associated, whereas ARF6 is often localized to the plasma membrane (13–17). Nevertheless ARFs can translocate to Golgi membranes upon GTP loading (13, 18) and to unspecified membranes following formyl-Met-Leu-Phe or M₃ receptor activation (10, 19, 20), so their precise intracellular location following stimulation is not clear.

The isoform of PLD that mediates ARF-dependent responses was thought for several years to be PLD1 because of its activation in vitro by ARF (and Rho and PKC) (5, 21). Nevertheless, recent evidence suggests that PLD2, and especially an amino-terminally truncated form of PLD2 can also be activated by ARF (22, 23). Both PLD1 and the truncated form of PLD2 are activated in vitro by ARF1 more effectively than by ARF6 (23). In contrast, PLD2 heterologously expressed in cells can be activated to a similar extent by constitutively active ARF1 and ARF6 (7). ARF-dependent PLD activity and GPCR-mediated PLD responses have been described in the plasma membrane compartment (24–26), although the identity of the isoform responsible was not clear. PLD1 is largely associated with Golgi and other intracellular membranes (27–29), but some is also associated with the plasma membrane (30–32), and the enzyme can be recruited to the plasma membrane during exocytosis (26, 33). In contrast, PLD2 is more generally associated with the plasma membrane (27, 34), although it too can be associated with Golgi structures (35).

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; TP, thromboxane A₂; i3, third intracellular loop; tm7, transmembrane domain 7; FLAG, D.YKDDDD epitope tag; HA, hemagglutinin; GST, glutathione S-transferase; sFM3, signal sequence-FLAG-tagged M3 receptor; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; ARF, ADP-ribosylation factor; [H]NMeqNB, [H]N-methylquinuclidinyl benzilate; [H]IalF, [H]inositol phosphate; [H]IγtBut, [H]phosphorylidobutanol; PMTx, P. multocida toxin; AEBSF, [4-(2-aminoethyl)benzene]sulfonyl fluoride; BFA, brefeldin A; GEF, GTP exchange factor; PDBu, phorbol 12,13-dibutyrate; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)]-1-propanesulfonic acid; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxyethylpropane-1,3-diol.

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M<sub>3</sub> Receptor-ARF Interactions

7.4 with 2 mM MgCl<sub>2</sub>, 2 μg/ml aprotinin, and aliquots were taken for protein assay (Coomassie binding method; Pierce). Homogenates were centrifuged at 12,000 × g for 30 min at 4 °C, and the pellet was washed twice more. For the binding assay, 1% bovine serum albumin was added. Ligand concentrations were varied from 20 pm to 2 nM, and nonspecific binding was defined by 1 μM NMe-atripione. After 4 h at 25 °C, an excess of ice-cold buffer was added, tubes were centrifuged, and the supernatant was aspirated from the pellet. Data were curve-fitted by nonlinear regression (Fig P. Elsevier-Biosoft, Cambridge, UK).

Cell surface specific binding of [3H]Hoxotomorin-M was measured to 1321N1 and sFM1 receptor-transfected COS7 cells in 12-well plates at 4 °C. Culture medium was replaced with phosphate-buffered saline containing 2 mM MgCl<sub>2</sub> and 1% bovine serum albumin and then plates were placed on ice. Ligands in 1% bovine serum albumin were put on ice to determine nonspecific binding, and was sampled and were incubated for 16 h at 4 °C to minimize internalization. Incubations were then quenched with excess ice-cold buffer and washed once. Icecold “acid strip” solution (0.2 M acetic acid, 0.5 μM NaCl) was added for 5 min to release surface-bound ligand. The internalization of specific [3H]Hoxotromorin-M binding sites into COS7 cells was measured at 37 °C over a time course of 0–50 min following the addition of ligand. Both ligand and NMe-atripione concentrations were as in the experiments carried out at 4 °C. Total and nonspecific binding levels were assessed at each time point. Following 5 min with cold acid strip solution to remove surface-bound ligand, cells were solubilized in 1% SDS, 1 M NaOH and then neutralized, to determine [3H]ligand in both cell surface and internalized compartments.

**Signal Transduction Assays—**Cellular [3H]Hniositol phosphate ([3H]InP) production (PLC activity) was measured in 12-well plates following labeling with 1 μM[3H]Hniositol for 18 h in serum-free medium. Agonist responses were measured usually over 30 min in the presence of 10 μM LiCl before cells were lysed in ice-cold 10 mM formic acid, and [3H]Hniositol phosphates were separated by ion exchange (38). Inhibitory agents and the LiCl were added 30 min and 15 min prior to agonist, respectively. [3H]Phosphatidylbutanol ([3H]PtdBut) production (PLD activity) was measured in 12-well plates following labeling with 1.5 μCi/well [3H]palmitate for 18 h in serum-free medium. It has been shown that the presence of 10 μM LiCl causes elevated [3H]PtdBut production (21). Agonist responses were measured usually over 30 min in the presence of 30 μM butan-1-ol. Assays were terminated, phospholipids were extracted into chloroform/methanol, and [3H]PtdBut was separated by thin layer chromatography (39). Inhibitory agents and the butan-1-ol were added 30 min prior to and immediately before agonist, respectively. In experiments with PtxM (40), agonist incubations were carried out over a total period of 4 h, with replacement of medium containing fresh PtxM and LiCl or butan-1-ol at 2 h. All data from signal transduction and ligand binding experiments are expressed as means ± S.E. from between 4 and 10 separate determinations.

**Immunoprecipitation of sFM1 Receptor—**In order to immunoprecipitate the sFM1 receptor with the M2 antibody (clone M2, 10 μg/ml), cells expressing the sFM1 receptor and either ARF1-αHA or ARF6-αHA were transiently transfected into COS7 cells. 72 h later, the cells were serum-deprived for 4 h. Cells were then exposed to carbocbl (20 μM) or no drug for 15 min and washed once in Hanks’ balanced salt solution before being solubilized in immunoprecipitation buffer (phosphate-buffered saline, pH 7.5, 1% CHAPS, 0.75% sodium deoxycholate, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM AEBBF, 2 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium molybdate, and 50 μg/ml soybean trypsin inhibitor (2 ml/l75 cm<sup>2</sup> flask for 1 h on ice). Carbocbl was readded where appropriate. Extracts were centrifuged at 12,000 × g for 15 min at 4 °C to remove particulate material and then dialyzed against Buffer AB (2 M sodium acetate, 2 M urea, 0.05 M Tris, pH 7.4) over 4 h. After centrifugation, the supernatant was removed to tubes containing either mouse monoclonal FLAG antibody (clone M2, 10 μg/ml; Sigma) or nonimmune mouse IgG (10 μg/ml; Sigma) with 40 μg/ml Protein G-Sepharose suspension, before rolling at 4 °C overnight. Beads were collected by centrifugation and washed 4 times with Buffer AB and then resuspended in Buffer AB containing 0.25% SDS, 5% mercaptoethanol, 20 mM Tris, pH 7.4) was added per ml of original supernatant. SDS-PAGE and electroblotting onto “Immobil-P” polyvinylidene difluoride membranes (Millipore Ltd., Watford, UK) were carried out using a Phastsystem apparatus (Amersham Biosciences). Western blots were carried out on the samples and original samples to detect immunoprecipitated proteins at different levels. The primary antibodies were rabbit polyclonal raised to the third intracellular loop of the M<sub>3</sub> receptor (41) (gift from Andrew Tobin) and rabbit polyclonal against the HA epitope tag (Santa Cruz Biotechnol, Autogen Bioclear Ltd., Calne, UK), followed by preabsorbed sec.
ondary antibodies conjugated to horseradish peroxidase (Chemicon International Ltd., Harrow, UK). Bands were visualized by ECL (Amersham Biosciences) and then measured by quantitative densitometry. In further experiments, an alternative procedure was used in which the sFMI receptor associated with ARF1-HA or ARF6-HA immunoprecipitates was measured by specific [3H]NMe-QNB binding. Cells treated with or without carbachol were solubilized in immunoprecipitation buffer with 10% glycerol, and precleared supernatants were immunoprecipitated with 2 μg/ml 12CA5 mouse monoclonal HA antibody (or nonimmune mouse IgG) for 90 min followed by Protein G-Sepharose for 40 min. This more rapid procedure was designed to minimize the possibility of any nonspecific interactions of the solubilized proteins. Immunoprecipitates were washed in immunoprecipitation buffer with 10% glycerol and then resuspended into [3H]NMe-QNB binding buffer (above) with 10% glycerol and 0.3 mg/ml sonicated phosphatidylinositol choline prior to ligand binding, as above.

Cell Surface Biotinylation—In some experiments, cell surface proteins were biotinylated using a membrane-impermeant reagent (biotinamidehexaconic acid 3-sulfosuccinimidyl ester sodium salt (Sigma); 1 mM for 2 h at 4°C). The reaction was quenched with 75 mM glycine (10 min at 4°C), and cells were washed in phosphate-buffered saline before returning to minimal essential medium and warming to 37°C. Cells were then stimulated with 20 μM carbachol (10 min) or control before solubilization. Extracts were incubated with monomeric avidin (1 mg/ml (1 h at 4°C) and washed in solubilization buffer before biotinylated proteins were eluted by incubation in 2 mM biotin for 30 min at 4°C. These supernatants were then subjected to immunoprecipitation with 12CA5 HA antibody (or nonimmune IgG control) and subsequently used in specific [3H]NMe-QNB binding assays, as above.

GST Fusion Protein Interaction Assays—The GST-M,3 (Arg[63]Gln[490]) construct in pGEX-4T-1 and the control GST-BKSTREX construct in pGEX-5X-1 were expressed in BL21-RIL bacterial cells, which were then grown up in standard 2x YT (yeast extract, tryptone, NaCl) medium with 2% glucose added. When the cells had reached an A600 of 0.6–0.8 units/ml, expression of the fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside for 3 h at 37°C. Cells were then pelleted by centrifugation and resuspended with Brefeldin A (Novagen, CN-Biosciences) for 10 min and again centrifuged. The supernatant, containing the GST fusion proteins, was added to glutathione-Sepharose beads (Amersham Biosciences). The beads were incubated with the bacterial supernatant for 20 min at room temperature to allow binding of the GST fusion proteins to the beads. The matrix formed was then washed extensively with phosphate-buffered saline and used immediately.

In order to provide cytosolic extracts enriched with various ARF constructs, transfected COS7 cells were homogenized in ice-cold extraction buffer (2 ml/175-cm² flask, 2 μg/ml aprotinin, 1 μg/ml AEBSF, 1 μM dithiothreitol, 2 μg/ml pepstatin, 1 μM sodium orthovanadate, 1 μM sodium fluoride and 10 μg/ml soybean trypsin inhibitor in phosphate-buffered saline). The cells were then homogenized (Ystral homogenizer; setting 3, 15 s) before being centrifuged (12,000 × g for 20 min at 4°C). The supernatant was aliquoted and stored at −40°C. ARF-HA-enriched extracts were incubated with the GST fusion protein affinity matrix in 250 μl of Buffer A (20 mM Tris-HCl, pH 7.5, 0.6 mM EDTA, 1 μM dithiothreitol, 70 mM NaCl, 0.05% Tween 80) for 90 min at 4°C with rolling. The beads were washed four times in Buffer A, and then the retained proteins were removed from the beads with 2× Laemmli buffer and applied to 20% homogenous Phastgels (Amersham Biosciences) and SDS-PAGE and subsequent Western blotting. Membranes were probed for HA immunoreactivity to monitor captured ARFs and for GST immunoreactivity to assess levels of fusion protein input (GST alone ~29 kDa, GST-M,3 ~49 kDa, and GST-BKSTREX ~55 kDa). Antibodies were rabbit polyclonal anti-HA and polyclonal anti-GST (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies and ECL were used as were used in the immunoprecipitation studies. Input levels of ARF-HA immunoreactivity in extracts were also monitored, and both fusion protein and ARF inputs were carefully balanced to ensure comparability between samples.

Subcellular Fractionation—Homogenates of sFMI receptor-transfected COS7 cells (in 175-cm² flasks), either control or treated with 200 μM carbachol for 10 min, were prepared and initially centrifuged at 1000 × g for 8 min to remove nuclei and unbroken cells. The remaining material was carefully fractionated through gradients of Percoll (Amersham Biosciences) under alkaline conditions designed to optimally separate endoplasmic reticulum, Golgi, and plasma membrane fractions (24). Fractions (0.5 ml) were downloaded from the bottom of the gradient by peristaltic pump (1 ml/min), and adjacent fractions were combined into Laemmli buffer for SDS-PAGE on 4–12% gradient Bis-Tris gels (Invitrogen) before immunoblotting for organelle marker proteins as well as for PLD1 and ARF1. The antibodies used were goat polyclonal anti-EEA1 (endoplasmic reticulum marker, Santa Cruz Biotechnology), mouse monoclonal anti-GM130 (Golgi marker; Transduction Laboratories, BD Biosciences, Cowley, UK), mouse monoclonal anti-Na+/K+ ATPase α1 subunit (plasma membrane marker; Upstate Biotech Ltd., Milton Keynes, UK), rabbit polyclonal anti-PLD1 (N-terminal region) (BIOSOURCE International Inc., Nivelles, Belgium), and sheep polyclonal anti-ARF1/3 (Upstate Biotech). For [3H]PtdInsP2 production experiments, each 175-cm² flask of cells was labeled with 150 μCi of [3H]palmitate in serum-free medium for 16 h prior to the experiment. Subcellular fractions were extracted with chloroform/methanol according to the standard PLD assay procedure, and [3H]PtdInsP2 was similarly separated by thin layer chromatography.

RESULTS

PLD Activation by Native M3 Receptors in 1321N1 Cells—Ligand binding studies with [3H]NMe-QNB demonstrated specific muscarinic binding sites in 1321N1 cells and sFMI receptor-transfected COS7 cells but not in mock-transfected COS7 cells. In 1321N1 cells, the KD of specific [3H]NMe-QNB binding were 0.26 ± 0.03 nM and 193 ± 27 fmol/mg protein, similar to previous work (42), which showed that the muscarinic receptors present were almost entirely of the M3 subtype. In sFMI receptor-transfected COS7 cells, the KD and Bmax of specific [3H]NMe-QNB binding were 0.58 ± 0.04 nM and 2.64 ± 0.47 pmol/mg protein. In pilot experiments with wild-type M3 receptor cDNA (lacking the signal sequence and FLAG tag), binding showed similar affinity but lower Bmax values.

In 1321N1 cells, the M3 agonist carbachol caused concentration-dependent increases in both [3H]PtdInsP2 and [3H]InsP3 production (Fig. 1a). The EC50 values for these PLD and PLC responses were similar, being 10.2 ± 2.0 and 8.1 ± 1.7 μM, respectively. The nicotinic cholinergic agonist 1,1-dimethyl-4-phenyl-piperazinium iodide caused no discernible increase in [3H]PtdInsP2 production through the range 3–100 μM (1.20 ± 0.17-fold of basal control at 100 μM 1,1-dimethyl-4-phenyl-piperazinium iodide, n = 4), indicating that nicotinic receptors made no significant contribution. The muscarinic partial agonist, aceclidine, activated PLD with a lower maximum response, in the order of 30% of that for carbachol (in line with its reported efficacy in PLC activation). 1321N1 cells also express the thromboxane A2 (TP) receptor, which like the M3 receptor is coupled to PLC activation via Gq/11 but contains an alternative efficacy in PLC activation). 1321N1 cells also express the thromboxane A2 (TP) receptor, which like the M3 receptor is coupled to PLC activation via Gq/11 but contains an alternative efficacy in PLC activation. 1321N1 cells also express the thromboxane A2 (TP) receptor, which like the M3 receptor is coupled to PLC activation via Gq/11 but contains an alternative efficacy in PLC activation (Fig. 1a) but with properties distinct from the basal receptor response.

The PLD response to 200 μM carbachol was inhibited in a concentration-dependent manner by brefendin A (BFA; a selective inhibitor of a subfamily of ARF GTP exchange factors (ARF-GEFs), known as BIG1/2) (43). The corresponding PLC response was unaffected (Fig. 1b). PLD responses to a low concentration of carbachol (10 μM) or to aceclidine (500 μM) showed similar BFA sensitivity to that with 200 μM carbachol, having IC50 values of 61.4 ± 9.5, 56.8 ± 11.1, and 55.5 ± 13.5 μM, respectively. In contrast, PLD activation by the TP receptor agonist U46619 was unaffected by BFA. The time course of PLD and PLC activation by carbachol in 1321N1 cells is shown in Fig. 1c. There was rapid desensitization of the PLD, but not the PLC response, over the times examined. BFA had no effect on the time course of PLC activation but diminished the initial rate and maximal extent of PLD activity, although the profile of desensitization was unaltered. Since PLD responses can occur downstream of PLC activation, we examined effects of the selective PLC inhibitor U73122. Fig. 1d shows that U73122...
had no effect on PLD responses of the M₃ receptor despite inhibiting PLC responses with an IC₅₀ value of 3.6 ± 1.9 μM. In contrast, PLD activation by the TP receptor agonist U46619 was readily inhibited by U73122 (IC₅₀ value of 3.4 ± 1.4 μM). In case G₁₁/M might play a role that was independent of PLC, we used the selective direct activator of G₁₁/M, PMTx, which was found to cause concentration-dependent activation of [³H]InsP₄ production (Fig. 2a). PMTx also caused [³H]PtdIns(4,5)P₂ production, and the response to a nearly maximally effective concentration (0.7 nM; 2.84 ± 0.36-fold of basal) was found to be inhibited readily by U73122 (IC₅₀ value of 3.1 ± 0.6 μM) but not by BFA (Fig. 2b). Pertussis toxin (100 ng/ml; 16 h) had no effect on carbachol-induced [³H]PtdIns(4,5)P₂ production (data not shown), indicating that G₁₁/M do not play a role here.

Since PKC and ARF can act synergistically to activate PLD (5), we further investigated a potential role for PKC in M₃ receptor responses. The selective PKC inhibitors CGP 41251, NPC-15437, chelerythrine chloride, and myristoyl-PKC were used the selective direct activator of G₁₁/M, PMTx, which was found to cause concentration-dependent activation of [³H]InsP₄ production (Fig. 2a). PMTx also caused [³H]PtdIns(4,5)P₂ production, and the response to a nearly maximally effective concentration (0.7 nM; 2.84 ± 0.36-fold of basal) was found to be inhibited readily by U73122 (IC₅₀ value of 3.1 ± 0.6 μM) but not by BFA (Fig. 2b). Pertussis toxin (100 ng/ml; 16 h) had no effect on carbachol-induced [³H]PtdIns(4,5)P₂ production (data not shown), indicating that G₁₁/M do not play a role here.

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PLD activation by the M₃ receptor may involve protein-tyrosine kinases in HEK 293 cells (9) but not 1321N1 cells (44), so we investigated the effects of the selective inhibitor of Src family tyrosine kinases, PP1 and the broad-spectrum tyrosine kinase inhibitors genistein and AG 213. None of these had any significant effect on carbachol-induced PLD activation in 1321N1 cells (Fig. 2d). However, in HEK 293 cells transiently transfected with the sFM₃ receptor construct, we found that carbachol-induced [³H]PtdIns(4,5)P₂ production was inhibited significantly by genistein and AG 213 with IC₅₀ values of 6.3 ± 1.3 and 15.8 ± 4.2 μM, respectively (n = 6).

Receptor-mediated PLD activation in some cells is sensitive to PI 3-kinase inhibitors (45), but we found no effect of wortmannin (1 μM) or LY 294002 (50 μM) on the concentration dependence, time course, or BFA sensitivity of carbachol-induced PLD activation in 1321N1 cells (data not shown).
Fig. 2. Evidence for the lack of major involvement of G_{q,11}, PKC, or tyrosine kinases in M_{3} receptor PLD responses in 1321N1 cells. The [^{3}H]PtdP根本就不产 production, whereas co-transfection with T31N-ARF1 gave a 2.80 ± 0.21-fold response, co-transfection with T27N-ARF6 gave a 3.26 ± 0.48-fold response, and co-transfection with a combination of the ARF1/6 mutant constructs resulted in a 3.68 ± 0.50-fold response (n = 5). Concentration dependence of PLD activation in response to PDBu was characterized by empty vector. The negative mutant ARF values were significantly less than carbachol alone, and the combination of ARF1/6 mutant constructs resulted in a 1.68 ± 0.05, Wilcoxon test). c, concentration dependence of the effects of PKC inhibitors on the PLD responses to 0.2 M carbachol (●, CGP 41251; ■, NPC-15437; ▲, chelerythrine chloride; ▼, myristoyl-PKC<sub>α/γ</sub> and to 30 nM PDBu (●, CGP 41251). The only statistically significant effect on PLD responses to PDBu was that of 30 μM chelerythrine chloride, whereas responses to PDBu were inhibited by 0.3–10 μM CGP 41251 (p < 0.05, Wilcoxon test). Bisindolylmaleimide I and calphostin C were not used because of known effects on the M₃ receptor and PLD, respectively. d, concentration dependence of the effects of tyrosine kinase inhibitors on the PLD responses to 200 μM carbachol (●, PP1; ■, genistein; ▲, AG 213) (n = 5).}

In contrast, we examined the PLD response to ATP (acting at native P₂U receptors). This was unaffected by BFA but was clearly reduced by the PKC inhibitor CGP 41251 and by transfection of T27N-ARF6 but not T31N-ARF1 (Fig. 3d). In contrast to the sFM₃ receptor, the native P₂U receptor thus appears to utilize PKC- and ARF6-dependent (but ARF1-independent) pathways for PLD activation. It seems unlikely that the difference between sFM₃ and P₂U receptors is due to heterologous expression because the findings with the sFM₃ receptor here mirror those obtained with the native M₃ receptor in 1321N1 cells (Figs. 1b and 2c). To corroborate this, we transfected COS7 cells with the N376D mutant 5-HT₂A receptor, which displays BFA-insensitive responses (in contrast to the wild type 5-HT₂A receptor, where BFA is effective) (12). PLD responses of the N376D mutant 5-HT₂A receptor were also significantly inhibited by the PKC inhibitors, CGP 41251 and bisindolylmaleimide I, but not by BFA, T31N-ARF1, genistein, or AG 213. Although T27N-ARF6 reduced responses by 20–25%, this did not reach statistical significance (Fig. 3d and data not shown).

Physical Association of ARF1/ARF6 with the sFM₃ Receptor—The question of whether ARF1 or ARF6 could participate in some form of direct complex with the receptor was investigated first by co-immunoprecipitation and second by in vitro interaction with a GST fusion protein of the M₃ receptor β3 domain. Fig. 4 shows co-immunoprecipitation data from COS7 cells co-transfected with sFM₃ receptor and wild type ARF1-HA or ARF6-HA. Input levels of ARF-HA and the efficiency of sFM₃ receptor pull-down were monitored to ensure balance between samples. In Fig. 4a, low levels of ARF1-HA and ARF6-HA immunoreactivity were associated with the sFM₃ receptor in basal conditions, apparently in excess of nonimmune IgG controls. Preincubation of cells with carbachol caused increased association of ARF1-HA but not ARF6-HA with the sFM₃ receptor, as monitored by densitometry of the immunoblots.
Carbachol at concentrations of 2 μM caused significant inhibition of the PLD responses to carbachol only in the presence of control vector ( ), wild type ARF1 ( ), wild type ARF6 ( ), T31N-ARF1 ( ), and T27N-ARF6 ( ). The negative mutant forms of both ARF1 and ARF6 significantly reduced PLD responses to carbachol at concentrations of 2–200 μM (p < 0.05, Wilcoxon test). b, shows the concentration dependence of PLC activation evoked by carbachol acting at the sFM3 receptor in the presence of control vector ( ), wild type ARF1 ( ), wild type ARF6 ( ), T31N-ARF1 ( ), and T27N-ARF6 ( ). The negative mutant forms of both ARF1 and ARF6 significantly reduced PLC responses to carbachol at concentrations of 2–200 μM (p < 0.05, Wilcoxon test). c, shows the concentration dependence of BFA effects on PLD responses to 200 μM carbachol in the presence of control vector ( ), T31N-ARF1 ( ), and T27N-ARF6 ( ). c, concentration dependence of BFA effects on PLD responses to 200 μM carbachol in the presence of control vector ( ), T31N-ARF1 ( ), wild type ARF1 ( ), T27N-ARF6 ( ), and T31N-ARF1 plus T27N-ARF6 ( ). d, shows the effects of the ARF-GEF inhibitor, BFA (150 μM; light gray columns) the PKC inhibitor, CGP 41251 (10 μM; medium gray columns), and transfected T31N-ARF1 (dark gray columns) or T27N-ARF6 (black columns) on basal [3H]PtdBut production or that in the presence of ATP (10 μM) acting at the native P2Y1 receptor or 5-HT (10 μM) acting at the sFM3 receptor-ARF1-HA association of ARF1-HA or ARF6-HA with GST fusion protein construct of the M3i3 domain, a control construct, or GST alone. The levels of each GST construct were shown to be similar by Coomassie Blue staining and by GST immunoreactivity. ARFs were supplied as enriched extracts from transfected COS7 cells, and binding was monitored by HA immunoblot. The data (which are representative of at least three separate experiments) demonstrate specific in vitro interaction of both ARF1-HA and ARF6-HA with the GST-M3i3 but not control constructs.

The Role of PLD1 and PLD2 in [3H]PtdBut Production by the sFM3 Receptor in COS7 Cells—Since both PLD1 and PLD2 can potentially be activated by ARFs, we investigated which PLD isoform was responsible for the ARF-mediated response of the receptor. Immunoblots for PLD isoforms in membranes of 1321N1, COS7, and HEK 293 cells showed that both PLD1 and PLD2 were present in each case (as in most cell types) (46) with a mean ratio of PLD1/PLD2 levels decreasing in the order COS7 > 1321N1 > HEK 293 (data not shown). Catalytically
inactive mutants of PLD1 (K898R-PLD1) and PLD2 (K758R-PLD2) were co-transfected with sFM₃ receptor plus ARF1-HA or ARF6-HA were stimulated with carbachol (20 μM, 15 min) or control prior to solubilization. Extracts were immunoprecipitated with FLAG antibody (or nonimmune IgG control) before SDS-PAGE and Western blotting. The left panel is from cells co-transfected with sFM₃ receptor and ARF1-HA; the right panel is from cells with sFM₃ receptor and ARF6-HA. In the top sections, the immunoprecipitate was probed with an antibody against the M₃ receptor i3 sequence. The middle sections show the input levels of immunoreactive ARF-HA in original extracts. The bottom sections show HA immunoreactivity associated with the immunoprecipitated receptor and indicate a carbachol-induced increase in the association of ARF1-HA but not ARF6-HA. The receptor runs as a broad band centered at about 90 kDa, diffuse because of glycosylation. ARF1-HA and ARF6-HA run at ≈20 kDa. A nonspecific band was seen at ≈30 kDa in all samples, which is likely to reflect nonspecific cross-reaction with immunoglobulins. These observations were typical of six separate experiments. b, results from an alternative procedure in which ARF-HA immunoprecipitates were probed for the presence of sFM₃ receptor by the measurement of specific [³H]NMe-QNB binding. Extracts from control mock immunoprecipitation with nonimmune IgG are shown for unstimulated cells (white columns) or following 20 μM carbachol for 5 min (dark gray columns). Corresponding anti-HA immunoprecipitates are shown from cells that were unstimulated (light gray columns) or carbachol-stimulated (black columns). Both ARF1-HA and ARF6-HA showed significantly increased association with specific [³H]NMe-QNB binding sites following carbachol compared with unstimulated or nonimmune IgG controls. Values are means ± S.E., n = 6, *, p < 0.05, Mann-Whitney U test. Input levels of both sFM₃ receptor and ARF1-HA/ARF6-HA in original cell extracts were shown to be matched between samples (data not shown). c, time course of association between ARF1-HA and the sFM₃ receptor as reflected by specific [³H]NMe-QNB binding. The ARF1-HA immunoprecipitates, but not nonimmune IgG controls (○ and □, respectively), showed a rapid time course of carbachol-induced increases in interaction (peaking at around 5 min and declining again to basal levels within 30 min).
evoked, sFM₃ receptor, and P₂ᵤ receptor-mediated responses. Wild type PLD2, but not the other constructs, caused a marked increase in basal [³H]PtdBut levels, matching reports of its constitutive activity (27). Responses to PDBu, carbachol, and ATP were all nonselectively increased. In contrast, wild-type PLD1 increased PDBu-evoked and sFM₃ receptor-mediated, but not P₂ᵤ receptor-mediated responses. PIM87-PLD1 caused a significant increase in sFM₃ receptor-mediated responses only, consistent with the idea that the role of PLD1 in sFM₃ receptor responses is independent of PKC.

Subcellular Trafficking of Components in M₃ Receptor PLD Activation—Since BFA disrupts the structural integrity of the Golgi apparatus at concentrations less than or equal to those used here (50), we asked whether altered trafficking of proteins needed for the signaling pathway, such as PLD itself, might contribute to the inhibitory effect of BFA. First, it is clear that a number of other GPCRs have PLD responses that are unaffected by BFA (Fig. 1b) (12). Second, when we compared the effects of BFA (Fig. 1b) with those of two further Golgi-disrupting agents, ilimaquinone and nocodazole (31, 35, 51), on PLD responses mediated by M₃ and TP receptors in 1321N1 cells, neither mimicked the effect of BFA receptor; nor did they affect responses to U46619 (30 μM) or PDBu (300 nM) (Fig. 1b; data not shown). [³H]PtdBut responses to 200 μM carbachol were 6.62 ± 0.46- and 5.72 ± 0.64-fold basal with ilimaquinone (25 μM for 30 min) and nocodazole (10 μM for 4 h), respectively, compared with values of 6.67 ± 0.34 and 3.31 ± 0.52 for carbachol alone and carbachol plus 100 μM BFA (n = 6). In the presence of ilimaquinone, the IC₅₀ for BFA was 72.1 ± 14.1 μM, similar to that in control conditions (Fig. 1b) and further suggesting that the effect of BFA on M₃ receptor PLD responses was distinct from any effects on Golgi structure.

To investigate whether endocytosis of the sFM₃ receptor might be necessary for its PLD responses, we utilized a dominant negative construct of dynamin 1, which reduces the internalization of agonist-occupied M₃ receptors (52, 53). Whereas transfection of K44A-dynamin 1 clearly reduced internalization of specific [³H]oxotremorine-M binding to the sFM₃ receptor in COS7 cells, carbachol-induced [³H]PtdBut production was unaltered, suggesting that endocytosis is not important for receptor PLD response (Fig. 7a). The internalization of specific [³H]oxotremorine-M binding was unaffected by BFA (150 μM for 30 min) or by transfection of either T31N-ARF1 or T27N-ARF6 (data not shown). To address more directly the possibility that sFM₃ receptor-mediated PLD activation might occur in endocytosing vesicles, we carried out subcellular fractionation of COS7 cell membranes after carbachol stimulation and analyzed the location of the [³H]PtdBut production. Alkaline Percoll gradients (24) were used to separate plasma membrane, Golgi, and endoplasmic reticulum fractions, characterized by immunoreactivity for Na⁺/K⁺ ATPase, GM130, and EEA1, respectively (Fig. 7b). Carbachol induced a large increase in [³H]PtdBut production in plasma membrane fractions, with a much smaller response being detected in Golgi and endoplasmic reticulum fractions.

The question of whether ARF and PLD proteins undergo translocation to the plasma membrane following stimulation with carbachol was addressed by immunoblots on the Percoll gradient fractions. Under basal conditions, ARF1 was distributed through plasma membrane and Golgi fractions, whereas PLD1 was detectable only in non-plasma membrane fractions (Fig. 7b). After carbachol stimulation, ARF1 and PLD1 became concentrated or newly detectable, respectively, in plasma membrane fractions, and this translocation was not prevented by the presence of BFA (Fig. 7b). ARF6 and PLD2 were detectable in plasma membrane fractions with or without carbachol (data not shown).

33.1 ± 8.9 μM, n = 10), the residual response in the presence of K898R-PLD1 was unaffected by BFA (Fig. 6c). We further examined the effects of T31N-ARF1 and T27N-ARF6 on responses in the presence of K898R-PLD1 or K758R-PLD2 expression. Fig. 6d shows that negative mutant ARF1 and ARF6 constructs significantly inhibited both control responses and those in the presence of K758R-PLD2. The residual [³H]PtdBut response in the presence of K898R-PLD1 was no longer sensitive to further inhibition by the negative mutant ARF1 construct but retained a small yet significant inhibitory effect of T27N-ARF6. These data suggest that the receptor uses an ARF1-mediated (BFA-sensitive) pathway to PLD1 and an ARF6-mediated (BFA-insensitive) pathway that can lead to either PLD1 or PLD2.

Fig. 6e demonstrates, in contrast, that K758R-PLD2 (but not K898R-PLD1) inhibits [³H]PtdBut responses of the P₂ᵤ receptor. Matching observations were made with the (similarly BFA-insensitive) N376D mutant 5-HT₁A receptor. The responses to 5-HT (10 μM) were 2.67 ± 0.36-, 2.89 ± 0.46-, and 1.28 ± 0.15-fold basal for the N376D-5-HT₁A receptor alone and that in the presence of K898R-PLD1 or K758R-PLD2, respectively. The inhibition due to K758R-PLD2 was statistically significant (p < 0.05, Mann-Whitney U test, n = 6). Fig. 6e also shows that PDBu-induced [³H]PtdBut production was attenuated by both K898R-PLD1 and K758R-PLD2, consistent with evidence that not only PLD1 (5–7) but also PLD2 (47–49) can be targeted by PKC. In addition, effects of wild type PLD1, wild type PLD2, and PIM87-PLD1 expression were compared on basal, PDBu-
agonist occupancy. PLC responses of the M3 receptor in both plugging to this pathway was not restricted to a particular level of concentrations and for a partial agonist, indicating that coupling to this pathway was not restricted to a particular level of agonist occupancy. PLC responses of the M3 receptor in both cell types were unaffected by BFA as were the PLD responses of control GPCRs, the TP receptor in 1321N1 cells and the P2U receptor and N376D mutant 5-HT2A receptor in COS7 cells. In contrast to the M3 receptor, which contains an NPXY motif in tm7, each of these contains the alternative DPXXY sequence, which is believed to prevent receptor coupling to BFA-sensitive PLD activation (12). PLD responses elicited by PMTx or U46619, but not by carbachol, were inhibited by the PLC inhibitor U73122, suggesting an indirect PLC-dependent route of PLD activation for the TP receptor but not the M3 receptor.

We considered further whether Ca\(^{2+}\) elevation or PKC activity might still play a role in M3 receptor PLD responses. 

**DISCUSSION**

A BFA-sensitive Route of PLD Activation for the M3 Receptor but Not Other GPCRs—The M3 muscarinic receptor shows BFA-sensitive activation of PLD when expressed as a native receptor in 1321N1 cells or heterologously in COS7 cells. Time course experiments showed rapid desensitization of M3 receptor PLD responses in 1321N1 cells as reported previously (44) and revealed that this was unaltered by BFA. BFA sensitivity of PLD responses was seen at low as well as high agonist concentrations and for a partial agonist, indicating that coupling to this pathway was not restricted to a particular level of agonist occupancy. PLC responses of the M3 receptor in both
mobilization does not appear to be an important mediator in 1321N1 (54) or HEK 293 cells (9) but the evidence for a role of PKC in M3 receptor PLD responses is equivocal. In 1321N1, but not HEK 293 cells, PKC down-regulation is reported to inhibit the M3 response (9, 55). However, the profound PKC activation involved in the procedure makes interpretation difficult. In apparent contrast, in M3 receptor-transfected HEK293 cells, co-transfection of the PKC activation-deficient mutant, PIM87-PLD1, yielded smaller PLD responses to carbachol than did excess wild type PLD1 (6), but it was not clear how this com-

![Fig. 7. Evidence that sFM3 receptor-mediated [3H]PtdBut production occurs at the plasma membrane of COS7 cells and involves agonist-induced translocation of mediator proteins to that compartment. a, the left panel shows the inhibitory effect of transfection with the K44A-dynamin 1 (dominant negative mutant) on internalization of [3H]oxotremorine-M (a hydrophilic agonist ligand) into an acid strip-resistant compartment. ○, cells with sFM3 receptor alone; ●, cells with sFM3 receptor and K44A-dynamin 1. The cell surface-specific binding of [3H]oxotremorine-M was unaffected (data not shown). The right panel shows [3H]PtdBut production in control cells in basal (white column) or carbachol (200 μM)-stimulated conditions (medium gray column) or carbachol-stimulated conditions (black column). sFM3 receptor-mediated PLD activation was unaltered by K44A-dynamin 1. b, subcellular distribution of membrane-associated basal (○) or carbachol (200 μM)-stimulated (●) [3H]PtdBut production in sFM3 receptor-transfected COS7 cells. Membranes were separated on Percoll gradients into fractions, which were characterized by immunoblot for the endoplasmic reticulum, Golgi, and plasma membrane markers (EEA1, GM130, and Na+/K+-ATPase, respectively). The response to carbachol was associated predominantly with plasma membrane fractions. c, the subcellular distribution of immunoreactivity for native PLD1 and ARF1/3 in membranes of cells under basal conditions or stimulated with carbachol (200 μM) or carbachol plus 150 μM BFA. Although the ARF antibody used cross-reacts with ARF3 as well as ARF1, the latter form is greatly predominant in cells. Under basal conditions, PLD1 was widely distributed, except in plasma membrane fractions, and ARF1/3 was present mainly in Golgi and plasma membrane fractions. Following carbachol stimulation, PLD1 immunoreactivity extended through to the plasma membrane, and ARF1/3 immunoreactivity became concentrated in plasma membrane fractions. BFA treatment did not disrupt carbachol-induced translocation of either PLD1 or ARF1/3.
pared with responses with native PLD alone. However, in experiments with the related M₁ receptor, PIM87-PLD1 facilitated the response to carbachol, compared with untransfected cells (7), suggesting that PKC-independent pathways to PLD were being utilized, as we found here with the M₃ receptor. Our observations with various PKC inhibitors, designed to block both catalytic and regulatory domains, provide no evidence to suggest a major contribution of PKC to the M₃ receptor PLD response in 1321N1 cells.

BFA inhibited M₃ receptor PLD responses here in 1321N1 and COS7 cells with IC₅₀ values of about 50 μM. BFA sensitivity of M₃ receptor PLD responses in HEK 293 cells has been reported previously but with some 2–3-fold lower potency (10), as we confirmed in transiently transfected HEK 293 cells (IC₅₀ of 157 ± 23 μM, n = 4). The lower potency in HEK 293 cells may reflect greater involvement of an alternative tyrosine kinase-dependent pathway. In A10 smooth muscle cells, PLD responses of angiotensin II and ET-1 receptors were strongly inhibited by BFA (56), whereas formyl-Met-Leu-Phe and ATP receptor responses in differentiated HL60 cells and Bradykinin and sphingosine 1-phosphate receptor responses in A549 adenocarcinoma cells were not (57, 58). The extent to which GPCRs demonstrate BFA-sensitive PLD responses in different cell types may well be influenced by the cellular content of mediators for particular pathways. The concentrations of BFA that selectively inhibit M₃ receptor PLD responses here exceed those needed to disrupt the integrity of Golgi membranes (50, 57, 58), but are similar to those that inhibit the ARF-GEFs, BIG1/2 (43). Considering whether disruption of Golgi traffic might play a role here, we confirmed that the cell surface expression of M₃ receptors and their PLC activation were unaffected by BFA (although these measures may not be very sensitive to acute disruption of trafficking). It is possible that PLD responses, but not other responses of GPCRs, may have a selective requirement for protein trafficking and thus may be selectively sensitive to Golgi disruption by BFA. Other GPCRs have clearly BFA-insensitive PLD responses, although theoretically they might generate their PLD responses by different mediators that are unaffected by disruption of the Golgi. However, the selective effect of BFA on M₃ receptor PLD responses was not mimicked by ilimaquinone and nocodazole, which also profoundly disrupt Golgi structure and function. Furthermore, we established that the subcellular location of carbachol-induced [³H]PtdBut production in sFM₃ receptor-containing COS7 cells was predominantly in the plasma membrane fraction and showed directly that whereas the response involved a movement of both PLD1 and ARF1 to this site, the translocation was not prevented by BFA. Similar observations were found using confocal microscopy (data not shown). Therefore, the effects of BFA on PLD responses of particular receptors appear to reflect a specific intervention in signal transduction rather than a general disruption of protein trafficking.

ARF1 and ARF6 Involvement in PLD Activation by the sFM₃ Receptor but Not Other GPCRs—We addressed the role of different subtypes of ARF in sFM₃ receptor PLD activation by transfection of either wild type ARF1 or ARF6 or their dominant negative constructs, T31N-ARF1 and T27N-ARF6 (14). Neither wild type ARF construct significantly affected PLD activation by carbachol, suggesting that the cellular content of endogenous ARFs is probably not a limiting factor. However, dominant-negative ARF1 and ARF6 constructs each inhibited PLD responses without modifying PLC responses. Effects of negative mutant ARF1 and ARF6 in combination were significantly greater than either alone, suggesting that the two ARF isoforms might each play a distinct role. Although negative or positive mutants of ARFs can disrupt Golgi and other vesicular trafficking (14, 59–62), we found that neither the levels of specific cell surface [³H]oxotremorine-M binding sites nor sFM₃ receptor PLC responses were affected by the ARF constructs here. In parallel with our observations, PLD responses of the angiotensin II and ET-1 receptors in A10 cells were inhibited by both T31N-ARF1 and T27N-ARF6 constructs (56). In contrast, we showed that the responses of two DPXY-containing mediators, the P₃A₄ receptor and the N376D mutant 5-HT₂₄ receptor, were unaffected by T31N-ARF1 but were clearly inhibited by T27N-ARF6 and PKC inhibitors (P₃A₄ receptor) or by PKC inhibitors alone (N376D mutant 5-HT₂₄ receptor). This suggests that ARF6 and PKC may be important in alternative pathways that underlie the BFA-insensitive [³H]PtdBut production seen with some GPCRs. The BFA sensitivity of M₃ receptor PLD responses was clearly preempted in the presence of dominant negative ARF1 but not ARF6, indicating that an ARF1-dependent pathway from the receptor, probably involving BIG1/2, is responsible for the sensitivity to BFA. Correspondingly, it has been shown that BIG1/2 can act as effective, BFA-sensitive ARF-GEFs for ARF1 but not ARF6 (43) and that in vivo functional effects of ARF6 are often BFA-insensitive (63, 64). The sFM₃ receptor PLD response in COS7 cells therefore appears to comprise at least two components: an ARF1-dependent BFA-sensitive pathway and an ARF6-dependent, BFA-insensitive pathway.

Physical Association of Both ARF1 and ARF6 with the M₃ Receptor through Its i3 Domain—Low levels of ARF1-HA and ARF6-6A were associated with sFM₃ receptor immunoprecipitates under basal conditions, whereas the amount of associated ARF1-6A but not ARF6-6A was clearly increased when cells were incubated with carbachol. In an alternative, rapid procedure where reduced nonspecific interactions were expected, we found that a small, carbachol-induced increase in ARF6-6A interaction with the receptor could be shown as well as that for ARF1-6A. The time course of carbachol-induced association of ARF1-6A with the receptor was similar to that for the increase in [³H]PtdBut production.

Using GST fusion proteins, we further investigated the interaction of ARF1 and ARF6 with the M₃ i3 domain, which is known to contain sites for interaction with heterotrimeric G proteins, arrestins, Gβγ, and the kinases GRK2 and CK1-α (37, 65–67). We showed previously that i3 domain splice variants of the PAC₁ receptor show marked differences in their BFA-sensitive activation of PLD but not other signaling responses (39), suggesting that M₃ i3 might be a good candidate site for ARF docking here. Specific binding of each ARF was demonstrated to the M₃i3 GST fusion protein but not control constructs.

PLD1 Involvement in PLD Activation by the M₃ Receptor but Not Other GPCRs—A catalytically inactive mutant of PLD1, but not PLD2, specifically attenuated carbachol-induced PLD responses in sFM₃ receptor-transfected COS7 cells. A component of the response remained unaffected by either negative mutant PLD1 or PLD2, perhaps due to limited ability of the constructs to access the necessary sites and compete effectively with endogenous PLD. Other studies on the PLD isozyme mediating GPCR responses have given varying results that may partly depend on cell type. In HEK 293 cells, M₃ receptor PLD responses were large in the presence of additional wild type PLD1, but not K898R-PLD1 (6, 7), although it was not clear whether K898R-PLD1 could reduce responses mediated by endogenous PLD. In our experiments, [³H]PtdBut responses of the sFM₃ receptor but not other GPCRs were selectively increased by both wild type PLD1 and PIM87-PLD1 and inhibited by K898R-PLD1, implicating PLD1 as the key effector. Our evidence that PKC inhibitors are ineffective on M₃ receptor PLD responses in 1321N1 or COS7 cells further supports the
idea that this connection between the receptor and PLD1 is independent of PKC. In contrast, \[^{3}H\]PtdBut responses of the P\(_{2Y}\) receptor and the N376D mutant 5-HT\(_{2A}\) receptor were inhibited by K758R-PLD2 but not K898R-PLD1. The addition of wild type PLD2, but not wild type PLD1, facilitated these responses, but effects were nonselective in that basal, M\(_3\) receptor, and PDBu-induced responses were all increased. Other reports also indicate a role of PLD2 in the responses of some GPCRs; in A10 cells, PLD responses to angiotensin II were inhibited by K758R-PLD2 but not K898R-PLD1 constructs (56), and in PC12 cells overexpressing PLD2, bradykinin receptors activate PLD2 through a PKC-dependent mechanism (47). Furthermore, the \(\mu\)-opioid receptor can elicit a BFA-sensitive PLD response in HEK 293 cells overexpressing PLD2, but not PLD1, that has been proposed to involve PLD2 association with the carboxyl-terminal tail domain of the receptor (68).

Relationship between M\(_3\) Receptor Interaction with a BFA-sensitive, ARF1-dependent Pathway and Its Activation of PLD1—When sFM\(_3\) receptor responses are partially reduced in the presence of K898R-PLD1 (but not K758R-PLD2), any further sensitivity to BFA or T31N-ARF1 is removed. This suggests that the inhibitory effect of BFA seen under normal conditions reflects primarily a pathway involving ARF1 and PLD1. T27N-ARF6 still caused some inhibition of responses in the presence of either K898R-PLD1 or K758R-PLD2, consistent with the idea that the ARF6-mediated component from the receptor may lead to either PLD1 or PLD2. However, we cannot definitively assign a PLD isoform to the ARF6-mediated component, because we are not sure that blockade by negative mutant PLD1 is complete and also negative mutant PLD2 did not significantly reduce control sFM\(_3\) responses.

The Subcellular Localization of sFM\(_3\) Receptor-mediated, ARF1-dependent Activation of PLD1—Since ARF1 (13–18, 69) and PLD1 (27–31) are not normally localized to a large extent at the plasma membrane, either the receptor or these mediators may need to undergo translocation to enable sFM\(_3\) receptor-induced \[^{3}H\]PtdBut production. One possibility might be that the receptor causes PLD activation in endocytosing vesicles following agonist stimulation. For some GPCRs, such a mechanism involving endocytosis of GPCR and/or transacti-
growth factor receptors, is important in their activation of extracellular signal-regulated kinase mitogen-activated protein kinase (70). PLD may be integral to these processes, since it participates in insulin-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation by generating phosphatidic acid in endocytosing vesicles and thereby recruiting the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, Raf-1 (71, 72). However, our findings seem inconsistent with such a mechanism here. Dominant negative mutant dynamin 1 inhibited endocytosis of agonist-occupied sFM\(_3\) receptors but had no effect on PLD activation, whereas cell surface biotinylation indicated that the great majority of sFM\(_3\) receptors associated with ARF1-HA immunoprecipitates had been present on the cell surface. Furthermore, sFM\(_3\) receptor-mediated \[^{3}H\]PtdBut production was associated with subcellular fractions containing plasma membrane rather than Golgi or endoplasmic reticulum. In addition, the content of native ARF1 and PLD1 in plasma membrane was clearly and selectively increased following carbachol stimulation. Corresponding results were found in confocal microscopy experiments (data not shown). These observations suggest that agonist-induced translocation of ARF1 and PLD1 to the plasma membrane, into the vicinity of sFM\(_3\) receptors, is important in enabling the receptor to signal via PLD.

In conclusion, the present experiments describe an ARF-dependent activation of PLD by the M\(_3\) muscarinic receptor that appears to be essentially independent of conventional routes of GPCR signaling. Instead, both ARF1 and ARF6 can associate physically with the receptor, as shown by co-immunoprecipitation and GST fusion protein experiments. Dominant negative constructs of ARF1/6 and PLD1/2 showed that the characteristic BFA-sensitive PLD activation shown by the M\(_3\) receptor appears to involve ARF1-mediated activation of PLD1 (Fig. 8). We demonstrated that agonist induces the translocation of ARF1 and PLD1 into the vicinity of the M\(_3\) receptor at the plasma membrane, where the response takes place. An additional ARF6-mediated component may involve PLD1 or PLD2, whereas other factors such as Rho family small G proteins could potentially also be involved. In contrast, examples of DPXYY-containing GPCRs, which lack BFA-sensitive PLD responses, utilize PKC (and also in some cases ARF6) to bring about activation of PLD2. The range of GPCR motifs and cell-
ular factors that determine receptor selectivity for these different pathways of PLD activation remains to be determined.

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