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Agonist-Induced Endocytosis of Muscarinic Cholinergic Receptors: Relationship to Stimulated Phosphoinositide Turnover

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Abstract: The ability of muscarinic cholinergic receptors to activate phosphoinositide turnover following agonist-induced internalization has been investigated. Incubation of SH-SY5Y neuroblastoma cells with oxotremorine-M resulted in a time-dependent endocytosis of both muscarinic receptors and α subunits of G_q and G_{11}, but not of isoforms of phosphoinositide-specific phospholipase C, into a subfraction of smooth endoplasmic reticulum (V_1). Agonist-induced increases in diacylglycerol mass and in ^{32}P-phosphatidate labeling, much of which was of the tetraenoic species, were also observed in the V_1 fraction, but these increases persisted when the agonist-induced translocation of receptors into the V_1 fraction was blocked. All enzymes of the phosphoinositide cycle were detectable in the V_1 fraction. However, with the exception of phosphatidylinositol 4-kinase, none was enriched when compared with cell lysates. Both ^{32}P-labeling studies and enzyme assays point to a very limited capacity of this fraction to synthesize phosphatidylinositol 4,5-bisphosphate, whereas the synthesis of phosphatidylinositol 4-phosphate is robust. These results indicate that endocytosed receptors do not appear to retain their ability to activate phosphoinositide turnover. The availability of the substrate for phospholipase C, phosphatidylinositol 4,5-bisphosphate, may be one factor that limits the activity of muscarinic receptors in this subcellular compartment. **Key Words:** $G_{\alpha q/11}$—Phospholipase C isozymes—Diacylglycerol—Phosphatidylinositol 4-phosphate 5-kinase—Lipid substrate availability—Phosphatidate.
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The internalization (sequestration) of receptors present at the cell surface into a membrane fraction that exhibits a lower density than that of the plasma membrane and is inaccessible to hydrophilic ligands is an adaptive response to the prolonged agonist occupancy of muscarinic cholinergic receptors (mAChRs) in neural tissues (Fiegenbaum and El-Fakahany, 1985; Harden et al., 1985; Thompson and Fisher, 1991). Although the agonist-induced endocytosis of cell surface receptors via caveolae and/or clathrin-coated pits has been reported (Garland et al., 1994; Roettger et al., 1995), results from our laboratory with human SH-SY5Y neuroblastoma indicate that mAChRs in this cell line are internalized predominantly via a clathrin-mediated mechanism (Slowiejko et al., 1996). SH-SY5Y cells express a relatively high density of mAChRs (20,000–30,000/cell) of the m_3 subtype, which couple to the $G_{q/11}$-mediated activation of phosphoinositide-specific phospholipase C (PLC), with the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), formation of inositol 1,4,5-trisphosphate, and rise in the concentration of intracellular Ca^{2+} (Lambert and Nahorski, 1990; Fisher et al., 1994; Slowiejko et al., 1994). In a previous study with the parent SK-N-SH cell line, we observed that when internalized mAChRs were prevented from recycling to the cell surface by the use of a low-temperature paradigm, there was a pronounced reduction in the magnitude of mAChR-stimulated phosphoinositide hydrolysis in response to the addition of either hydrophilic or lipophilic agonists (Thompson and Fisher, 1991). These results led us to the conclusion that mAChRs localized at the plasma membrane were preferentially coupled to PLC activation. One possible explanation for these results is that the agonist-induced phosphorylation of cell surface mAChRs (Tobin and Nahorski, 1993;

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Abbreviations used: CDP-DAG, cytidine diphosphodiacylglycerol; DAG, *sn*-1,2-diacylglycerol; ECL, enhanced chemiluminescence; mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; Oxo-M, 2-butyn-1-ammonium, *N,N,N*-trimethyl-4-(2-oxo-1-pyrrolidinyl) iodide or oxotremorine-M; PA, phosphatidic acid; PBS-T, phosphate-buffered saline containing Tween 20; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; PVDF, polyvinylidene fluoride; QNB, quinuclidinyl benzilate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Tobin et al., 1996) results in both their internalization and their uncoupling from PLC. However, the relationship between phosphorylation of the mAChR, receptor internalization, and functional uncoupling has yet to be established, and other explanations for the apparent inability of endocytosed mAChRs to activate PLC need to be considered. In fact, the issue of whether the internalization of receptors results in their uncoupling from PLC remains to be established. In this context, it should be noted that, in addition to mAChRs, both G_q and PLC are present in subcellular fractions derived from the endocytic pathway (Harden et al., 1985; Martín et al., 1991; Svoboda and Milligan, 1994). Moreover, in these same fractions, the addition of guanine nucleotides can regulate both PLC activity and the affinity with which agonists bind to mAChRs (Harden et al., 1985; Martín et al., 1991). In addition, dopamine D_1, dopamine D_2, and adenosine A_1 receptors have been shown to retain their ability to modulate adenylyl cyclase activity when present in clathrin-coated vesicle fractions (Gonzalez-Calero et al., 1990; Ozaki et al., 1994). Evaluation of whether phosphoinositide-linked receptors can activate PLC at subcellular locations other than at the plasma membrane is made difficult by the fact that the extent of agonist-stimulated PLC activity, even if detectable, is substantially reduced when monitored in cell-free preparations. In addition, to date there has been no systematic study of the enrichment of the individual components of the phosphoinositide-signaling pathway (i.e., G_q, PLC, and the enzymes of phosphoinositide turnover) in the cellular compartment into which cell surface receptors are endocytosed.

In the present study, we have addressed the functional status of internalized mAChRs in SH-SY5Y cells after the isolation of a subcellular fraction (V_1) that is enriched in endocytosed receptors. The following questions have been addressed: (1) Do other components of the phosphoinositide-signaling pathway also undergo endocytosis in response to mAChR activation? (2) Are all of the enzymes necessary for operation of the phosphoinositide cycle present in this endocytic fraction? The results indicate that although all enzymes of the phosphoinositide cycle can be detected in V_1, most are not enriched, and the ability of this fraction to synthesize PIP_2 is very limited. However, the α subunits of G_q and G_{11} (but not PLC or other enzymes of phosphoinositide turnover) undergo endocytosis along with the mAChR after exposure of the cells to muscarinic agonists. Although increases in *sn*-1,2-diacylglycerol (DAG) mass and [^{32}P]phosphatidic acid ([^{32}P]PA) labeling are observed in the subcellular fraction enriched in endocytosed mAChRs, the increases in lipid turnover persist when receptor translocation is blocked. These results suggest that when mAChRs are endocytosed they lose their ability to activate PLC and that a determining factor in this loss of responsiveness may be a limited substrate availability. A preliminary account of part of this study has previously been reported (Sorensen et al., 1997).

MATERIALS AND METHODS

[*N-methyl*-^{3}H]Scopolamine methyl chloride ([^{3}H]NMS; 87 Ci/mmol), [^{3}H]scopolamine (74.9 Ci/mmol), [γ-^{32}P]-ATP (6,000 Ci/mmol), [^{3}H]ouabain (49.0 Ci/mmol), [^{3}H]inositol (110 Ci/mmol), peroxidase-conjugated sheep anti-mouse IgG, and detection reagents for enhanced chemiluminescence (ECL) were purchased from Amersham (Arlington Heights, IL, U.S.A.). [^{3}H]Phosphatidylinositol 4-phosphate ([^{3}H]PIP; 1 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB; 45.4 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Phosphatidylinositol (PI), PIP, DAG, PA, 2′-deoxycytidine 5′-triphosphate, ATP, *myo*-inositol, cytochrome *c*, NADPH, atropine, and pilocarpine were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). 2-Butyn-1-ammonium, *N,N,N*-trimethyl-4-(2-oxo-1-pyrrolidinyl) iodide (oxotremorine-M, Oxo-M) was purchased from Research Biochemicals International (Natick, MA, U.S.A.). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY, U.S.A.) and Sarstedt (Newton, NC, U.S.A.). Powdered Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GIBCO (Grand Island, NY, U.S.A.) and BioWhittaker (Walkersville, MD, U.S.A.). Polyclonal antibodies to PLC-β1, PLC-β3, $G_{\alpha q/11}$, G_β, $G_{\alpha s}$, and peroxidase-conjugated goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti–PLC-γ1 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Cardiolipin was from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Recombinant DAG kinase (*E. coli*) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Cytidine diphosphodiacylglycerol (CDP-DAG) was obtained from Doosan-Serdary Research (Englewood Cliffs, NJ, U.S.A.). 1-Methyl-3-nitro-1-nitrosoguanidine was from Aldrich Chemical (Milwaukee, WI, U.S.A.). Human SH-SY5Y neuroblastoma cells were obtained from Dr. June Biedler (Sloan-Kettering Institute, New York, NY, U.S.A.).

Cell culture conditions

SH-SY5Y cells (passage nos. 68–75) were routinely grown in tissue culture flasks (75 cm^2/250 ml) in 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% (by volume) fetal calf serum. Cells were grown for 7–14 days at 37°C in an atmosphere consisting of 10% CO_2 and 90% humidified air. Cells were isolated after aspiration of the medium and incubation with a modified Pucks D_1 solution (Honneger and Richelson, 1976). Cells were then resuspended in buffer A (142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 5.6 m*M* D-glucose, and 30 m*M* HEPES, pH 7.4).

Subcellular fractionation

A modified procedure of the method of Strader et al. (1984) was used to obtain crude plasma membrane (P_1) and "light" membrane (V_1) fractions as previously described (Slowiejko et al., 1996). The high-speed supernatant fraction (S_2) was also retained for assay. In some experiments, the V_1 fraction was applied to a discontinuous density gradient consisting of 60, 48, and 35% (wt/vol) sucrose and centrifuged at 150,000 *g* for 90 min. Material collecting at

the 0–35% (V_2), 35–48% (V_3), and 48–60% (V_4) interphases was removed, diluted with 10 m*M* Tris buffer (pH 7.4) containing 1 m*M* EDTA and centrifuged at 90,000 *g* for 90 min.

Radioligand binding

Whole cell lysates and subcellular fractions of SH-SY5Y cells were resuspended in KGEH buffer (139 m*M* potassium glutamate, 4 m*M* $MgCl_2$, 10 m*M* EGTA, and 30 m*M* HEPES, pH 7.4). Fractions were then incubated with either 6 n*M* [^{3}H]NMS, 1 n*M* [^{3}H]QNB, or 6 n*M* [^{3}H]scopolamine at 37°C for 90 min. These concentrations of radioligands are sufficient to occupy >90% of mAChR sites in lysates and subcellular fractions. Nonspecific binding was determined as that unaffected by inclusion of 25 μM atropine. Reactions were rapidly terminated by filtration through Whatman GF/B glass–fiber filters and radioactivity was determined after the addition of 5 ml of Universol scintillation fluid.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Aliquots (25 μg) of whole cell lysates or subcellular fractions of SH-SY5Y cells were boiled in SDS-PAGE sample buffer for 5 min and electrophoresed through 10% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.) and processed for immunoblot analysis. It should be noted that the separation of some protein isoforms (including $G_{\alpha q}$ and $G_{\alpha 11}$) is facilitated by the presence of sodium tetradecyl sulfate present in commercially available SDS (see Siegel and Desmond, 1989).

Western blot analysis

Nonspecific binding sites were blocked in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin for 1 h at room temperature. Primary antibodies were diluted in blocking solution (final concentration, 0.1–0.5 μg/ml) and incubated with the membranes for 1 h. Excess primary antibody was removed by washing the membranes three times in PBS-T. The blots were then incubated in the appropriate peroxidase-conjugated secondary antibody diluted in PBS-T (1:10,000) for 1 h and subsequently washed three more times in PBS-T. Immunoreactive proteins were detected by ECL. Membranes were reprobed after stripping in 0.1 *M* Tris-HCl (pH 8.0), 2% SDS, and 100 m*M* β-mercaptoethanol for 30 min at 52°C. The blots were rinsed two times in PBS-T and processed as above with a different primary antibody. Quantitative analysis of autoluminograms was performed by computer-assisted imaging densitometry (MCID, Imaging Research, St. Catharines, Ontario, Canada). The assignment of specific bands as $G_{\alpha q}$ and $G_{\alpha 11}$, which were detected with the same antibody, was performed based on reported electrophoretic mobilities.

^{32}P-Phospholipid labeling

Aliquots of P_1 and V_1 fractions (100–400 μg of protein) were resuspended in 150 μl of KGEH buffer (free Ca^{2+}, 50 n*M*). Reactions were initiated by the addition of 100 μl [^{32}P]ATP (final concentration, 50 μM; sp. act. 2.5–4.5 $\times 10^6$ cpm/nmol) and allowed to continue for the times indicated (0–5 min at 37°C). In some experiments, the concentration of ATP was increased to 200 μM to ensure that its availability was not a rate-limiting factor. Reactions were terminated by the addition of 0.75 ml $CHCl_3/CH_3OH$ (1:2). Lipids were extracted, separated, and quantitated as previously described (Fisher et al., 1984).

Argentation TLC

PA formed from the ^{32}P-phospholipid-labeling experiment was isolated and extracted from the silica gel with three 1.0-ml aliquots of $CHCl_3/CH_3OH/HCl$ (1:2:0.05). The combined extracts were washed with 2.0 ml $CHCl_3/CH_3OH/H_2O$ (3:48:47) and blown to dryness under N_2. PA was methylated with excess diazomethane (Fales et al., 1973) and separated according to degree of unsaturation on 40% (wt/wt) silver nitrate–impregnated TLC plates as outlined by Van Rooijen et al. (1985).

DAG mass

The mass of DAG present in aliquots of intact cells and P_1 or V_1 fractions (100–300 μg of protein) was monitored by the method of Preiss et al. (1986), after initial lipid extraction (Fisher and Agranoff, 1980). DAG standards (100–1,000 pmol) were routinely run in parallel.

DAG kinase

Activity was monitored by the method of Besterman et al. (1986), with the exceptions that the incubation time was reduced to 2 min and the concentration of ATP increased to 300 μM.

PLC

Activity was monitored using [^{3}H]PIP as substrate. Assays were performed in a final volume of 100 μl containing 10 m*M* HEPES (pH 7.4), 120 m*M* KCl, 10 m*M* NaCl, 2 m*M* EGTA, 2.2 m*M* $CaCl_2$, 6.0 m*M* $MgCl_2$, and 0.5% sodium cholate. The final concentration of [^{3}H]PIP was 50 μM (~20,000 dpm) and the lipid was added after having been blown down under N_2 and sonicated in 10 m*M* HEPES (pH 7.4) buffer. Reactions were allowed to proceed for 5 min at 30°C and were terminated by the addition of 0.375 ml $CHCl_3/CH_3OH/HCl$ (20:40:1), followed by 0.125 ml $CHCl_3$ and 0.125 ml 0.1 *M* HCl. Radioactivity was then quantitated after the addition of 5 ml of Universol scintillation fluid to a 0.2-ml aliquot of the upper phase.

CDP-DAG synthase

Activity was measured as previously described by Sparrow and Raetz (1985) and Kelley and Carman (1987). Aliquots of whole cell lysates, and P_1 or V_1 fractions (40–100 μg of protein), were incubated for 10 min at 37°C in 50 m*M* Tris-maleate buffer (pH 6.5), 1 m*M* PA, 20 m*M* $MgCl_2$, 1 mg/ml bovine serum albumin, and 1 m*M* [γ-^{32}P]dCTP (22,000 cpm/nmol) in a final volume of 200 μl. Reactions were terminated by the addition of 1.7 ml $CHCl_3/CH_3OH$ (1:2) and lipids extracted after the addition of 0.5 ml 1.2 *M* HCl and 1.0 ml $CHCl_3$. The lower phase was washed twice with 2 ml of acidified Folch upper phase and radioactivity present in 1.0-ml aliquots of the organic phase determined.

PI synthase

Activity was monitored by the method of Fischl and Carman (1983), as modified by Heacock et al. (1993), with the exceptions that $MnCl_2$ was excluded and the final concentration of inositol was 1 m*M* (22,000 cpm/nmol).

PI and PIP kinase

Activities were measured as previously described by MacDonald et al. (1987).

Other measurements

NADPH–cytochrome *c* reductase activity was measured spectrophotometrically at room temperature by the method of Sotocassa et al. (1967). [^{3}H]Ouabain binding was determined by the method of Svoboda and Milligan (1994). Protein content was measured with the Pierce BCA protein assay reagent (Rockford, IL, U.S.A.).

Data analysis

Results given are mean ± SEM values for the number (n) of separate experiments performed. For every parameter monitored, duplicate or triplicate replicates were obtained. Student's two-tailed *t* tests were used to evaluate the statistical differences of the means of paired or unpaired sets of data.

RESULTS

Agonist-induced subcellular redistribution of cell surface mAChRs

Evaluation of the functional status of internalized mAChRs required the isolation of a subcellular fraction from SH-SY5Y cells in which sequestered mAChRs were concentrated and one that yielded sufficient material for biochemical studies. Initial attempts to obtain such a fraction after the application of hypotonic cell lysates to continuous or discontinuous sucrose density gradients were unsuccessful due to the limited degree of enrichment of mAChRs obtained in any single fraction. A more effective separation of internalized mAChRs was obtained using a modification of the differential centrifugation procedure of Strader et al. (1984). Using this approach, a redistribution of mAChRs from a crude plasma membrane fraction (P_1) into a light vesicular fraction (V_1), which sedimented at 200,000 *g* for 90 min was observed when intact SH-SY5Y cells were incubated with Oxo-M for 30 min at 37°C. Whereas only 3% of total mAChRs recovered were located in the V_1 fraction obtained from quiescent cells, this value increased to 16% upon exposure of cells to Oxo-M (500 ± 73% of control, n = 5, Table 1). This appearance of mAChR binding sites in the V_1 fraction was accompanied by a corresponding loss of [^{3}H]QNB binding sites from the P_1 fraction. Marker analysis indicated that <2% of [^{3}H]ouabain binding (a measure of plasma membrane Na^+,K^+-ATPase) was recovered in the V_1 fraction and that this distribution was unaltered by the addition of agonist. Although the P_1 fraction possessed the majority of the NADPH–cytochrome *c* reductase activity (a marker for smooth endoplasmic reticulum; Table 1), significant amounts of this enzyme (35–41%) were recovered in the V_1 fraction, indicating that the latter represents, in part at least, a subfraction of the smooth endoplasmic reticulum. No subcellular redistribution of NADPH–cytochrome *c* reductase activity occurred after exposure of the cells to Oxo-M. In some experiments, the V_1 fraction was applied to a discontinuous sucrose gradient consisting of 35, 48, and 60% sucrose and centrifuged at 150,000 *g* for 90 min to further fractionate V_1. After density gradient centrifugation, >90% of mAChRs and protein recovered were located at the 0–35% sucrose interface (V_2), an equilibrium density at which internalized mAChRs, β_2-adrenergic, and transferrin receptors have previously been observed (Harden et al., 1985; Lohse et al., 1990; Sillence and Downes, 1993). Because the agonist-induced increases in mAChR density in the V_2 fraction obtained from SH-SY5Y cells (430–650% of control) were comparable with those observed for the V_1 fraction (see Table 1), the latter was used for all subsequent experiments.

In intact cells, when monitored as the loss of [^{3}H]-NMS binding sites, 30–40% of cell surface mAChRs are sequestered upon the addition of Oxo-M (Slowiejko et al., 1996), whereas <20% of receptors are recovered in the V_1 fraction by using the fractionation paradigm. Thus, to determine whether the subcellular redistribution of mAChRs into the V_1 fraction was

TABLE 1. *Subcellular distribution of mAChRs and marker enzymes in control and Oxo-M–stimulated cells*

	Specific activity of lysate	Percentage of total recovered			
		P_1	V_1	S_2	% of recovery
Control					
[^{3}H]QNB binding (fmol/mg of protein)	289 ± 82	97 ± 1	3 ± 1	ND	84 ± 6
[^{3}H]Ouabain binding (pmol/mg of protein)	2.33 ± 0.16	98 ± 1	2 ± 1	ND	82 ± 13
NADPH–cytochrome *c* reductase (nmol/mg of protein/min)	7.5 ± 0.7	65 ± 2	35 ± 2	ND	106 ± 13
Protein	—	48 ± 1	19 ± 1	33 ± 1	103 ± 3
Oxo-M					
[^{3}H]QNB binding (fmol/mg of protein)	262 ± 72	84 ± 2[a]	16 ± 2[a]	ND	79 ± 4
[^{3}H]Ouabain binding (pmol/mg of protein)	2.38 ± 0.17	98 ± 1	2 ± 1	ND	74 ± 14
NADPH–cytochrome *c* reductase (nmol/mg of protein/min)	7.3 ± 0.97	59 ± 2	41 ± 2	ND	114 ± 15
Protein	—	46 ± 2	21 ± 2	35 ± 2	96 ± 7

SH-SY5Y cells (~100 mg of protein) were incubated in the absence (Control) or the presence (Oxo-M) of 1 m*M* Oxo-M for 30 min and then hypotonically lysed and fractionated as described in Materials and Methods. Cell lysates, P_1, V_1, and S_2 fractions were assayed for [^{3}H]QNB, [^{3}H]ouabain binding, protein content, and NADPH–cytochrome *c* reductase activity. Results shown are mean ± SEM values for four or five separate experiments. ND, not detectable.

[a] Different from control, $p < 0.05$.

representative of sequestration events monitored in intact cells, the characteristics of this agonist-mediated translocation of mAChRs were further examined. The appearance of mAChRs in the V_1 fraction was time dependent with the onset detectable within 5 min of Oxo-M addition and the maximal effect observed after 30–60 min ($t_{1/2}$ = 12 min; Fig. 1A), results comparable with those previously observed for receptor sequestration in intact SH-SY5Y cells (Fisher et al., 1994; Slowiejko et al., 1994). The appearance of mAChRs in the V_1 fraction also displayed a similar dependence on agonist concentration and efficacy to that observed for sequestration in intact cells. Thus, half-maximal translocation was observed at a 3–10 μM concentration of Oxo-M (data not shown), and addition of the partial agonist pilocarpine (1 mM) elicited a significantly smaller redistribution of mAChRs than that observed for Oxo-M (139 ± 3% of control, n = 4; Fig. 1A). Receptors recovered in the V_1 fraction after Oxo-M addition were differentially recognized by muscarinic antagonists. The lipophilic ligands [^{3}H]QNB and [^{3}H]scopolamine consistently detected more mAChR sites than the hydrophilic ligand [^{3}H]NMS, even when

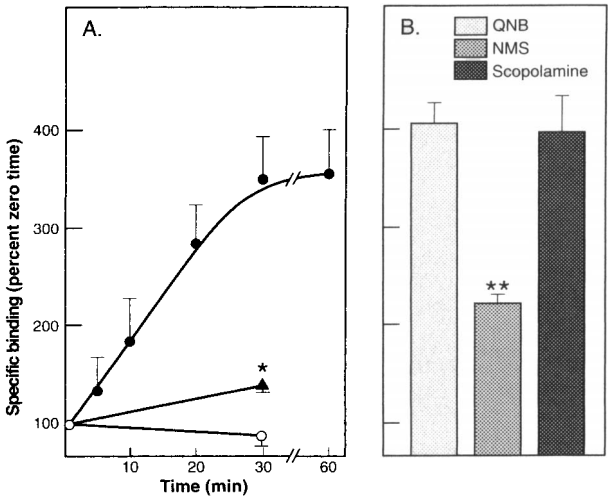

FIG. 1. Oxo-M pretreatment of SH-SY5Y cells results in the translocation of mAChRs into the V_1 fraction. **A:** SH-SY5Y cells were preincubated with either buffer A (○), 1 mM pilocarpine (▲), or 1 mM Oxo-M (●) at 37°C for the times indicated, the reactions terminated by the addition of ice-cold buffer A and the V_1 fractions obtained from hypotonic cell lysates as described in Materials and Methods. mAChRs present in the V_1 fraction were then monitored by means of [^{3}H]QNB binding. Values are expressed as the specific binding of [^{3}H]QNB (percentage of zero time values). Results shown are mean ± SEM values for three or four separate experiments. **B:** Cells were preincubated with 1 mM Oxo-M for 30 min and the V_1 fraction isolated after cell lysis and differential centrifugation. The specific binding of [^{3}H]QNB, [^{3}H]NMS, and [^{3}H]scopolamine to mAChRs in the V_1 fraction was then measured. Values are expressed as specific binding (percentage of zero time values). Results shown are mean ± SEM values for four separate experiments. *Different from Oxo-M, $p < 0.05$; **different from either [^{3}H]QNB or [^{3}H]-scopolamine binding, $p < 0.05$.

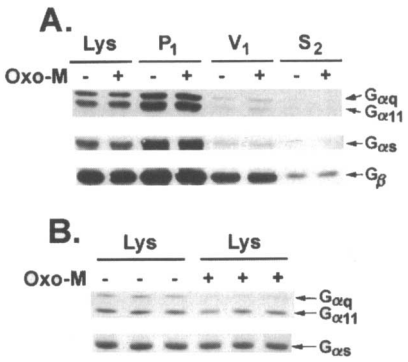

FIG. 2. Western blot analysis of the α subunits of G_q, G_{11}, and G_s and of G_β in subcellular fractions derived from control or Oxo-M–pretreated cells. **A:** Suspensions of SH-SY5Y cells were incubated in the presence of either buffer A(−) or 1 mM Oxo-M (+) for 30 min, the cells lysed, and subcellular fractions isolated as described in Materials and Methods (Lys = whole cell lysates). Equivalent aliquots (25 μg) of cellular protein from whole cell lysates or subcellular fractions were electrophoresed through 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were then immunoblotted for $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha s}$, or G_β (at primary antibody concentrations of 0.5 μg/ml for $G_{\alpha q}$/$G_{\alpha 11}$ and $G_{\alpha s}$ and 0.1 μg/ml for G_β) as described in Materials and Methods. Results shown are representative of those obtained in four separate experiments. **B:** SH-SY5Y cells, grown in culture dishes, were incubated either in the absence (control) or presence of 1 mM Oxo-M for 24–48 h. Cells were then lysed and 25-μg aliquots taken for SDS-PAGE and western blot analysis. Results shown are representative of those obtained in four separate experiments. [^{3}H]QNB binding in Oxo-M–treated cells was reduced by 52 ± 4% under these conditions (n = 4).

assayed at 37°C (Fig. 1B). Taken collectively, the results indicate that although fewer mAChRs are recovered in the V_1 fraction than are "lost" from the surface of intact SH-SY5Y cells, the characteristics of mAChR translocation into the V_1 fraction resemble those observed for receptor sequestration in intact cells in terms of kinetics, agonist concentration dependence, and agonist efficacy. Furthermore, it is apparent that the V_1 fraction is enriched in endocytosed mAChRs that are readily recognized by lipophilic, but not hydrophilic, ligands.

$G_{\alpha q}$ and $G_{\alpha 11}$ are endocytosed with the mAChR

The subcellular distribution of $G_{\alpha q/\alpha 11}$ and $G_{\alpha s}$ in control and Oxo-M–pretreated cells was determined by western blot analysis and quantitative densitometry (Fig. 2A). In control cells, all three subunits analyzed were significantly enriched in the P_1 fraction compared with whole cell lysates (359 ± 50, 236 ± 7, and 219 ± 31%, for $G_{\alpha q}$, $G_{\alpha 11}$, and $G_{\alpha s}$, respectively, n = 4). In contrast, there was little immunoreactivity associated with $G_{\alpha q}$, $G_{\alpha 11}$, or $G_{\alpha s}$ in the V_1 fraction (13 ± 6, 11 ± 5, and 21 ± 9% of lysate values, respectively, n = 4). The α subunits of all three G proteins were essentially absent from the S_2 fraction. Prior exposure of the cells to Oxo-M for 30 min resulted in an increase in $G_{\alpha q}$ and $G_{\alpha 11}$ immunoreactivity in the V_1 fraction

TABLE 2. *Subcellular distribution of enzymes of phosphoinositide turnover in control and Oxo-M–stimulated cells*

	Specific activity of lysate (nmol/mg/min)	Relative specific activity			Percentage of total recovered			% of recovery
		P_1	V_1	S_2	P_1	V_1	S_2	
PLC								
Control	6.63 ± 1.52	0.72 ± 0.14	0.29 ± 0.09	2.64 ± 0.37	19 ± 4	4 ± 1	77 ± 6	114 ± 6
Oxo-M	7.97 ± 2.79	0.75 ± 0.25	0.42 ± 0.10	2.59 ± 0.46	19 ± 4	5 ± 1	76 ± 4	119 ± 10
DAG kinase								
Control	0.57 ± 0.06	0.36 ± 0.08	0.58 ± 0.05	1.37 ± 0.30	27 ± 7	16 ± 2	57 ± 10	69 ± 7
Oxo-M	0.62 ± 0.03	0.45 ± 0.09	0.59 ± 0.10	1.42 ± 0.23	26 ± 7	15 ± 3	58 ± 9	67 ± 3
CDP-DAG synthase								
Control	1.39 ± 0.16	1.50 ± 0.19	0.81 ± 0.05	0.04 ± 0.01	81 ± 2	17 ± 2	2 ± 0	97 ± 11
Oxo-M	1.34 ± 0.21	1.60 ± 0.38	0.93 ± 0.05	0.03 ± 0.01	78 ± 2	21 ± 2	2 ± 0	90 ± 8
PI synthase								
Control	0.68 ± 0.28	2.02 ± 0.19	0.73 ± 0.15	0.02 ± 0.01	86 ± 2	13 ± 3	2 ± 1	116 ± 4
Oxo-M	0.62 ± 0.27	2.31 ± 0.42	0.86 ± 0.17	0.02 ± 0.01	85 ± 4	13 ± 5	2 ± 1	123 ± 27
PI kinase								
Control	0.58 ± 0.12	1.05 ± 0.16	2.21 ± 0.19	0.42 ± 0.07	49 ± 2	39 ± 1	12 ± 2	103 ± 10
Oxo-M	0.59 ± 0.12	1.27 ± 0.15	2.09 ± 0.15	0.41 ± 0.06	50 ± 3	37 ± 2	13 ± 3	101 ± 7
PIP kinase								
Control	0.06 ± 0.02	1.47 ± 0.22	0.40 ± 0.08	0.67 ± 0.36	67 ± 8	12 ± 3	21 ± 11	80 ± 11
Oxo-M	0.04 ± 0.02	1.74 ± 0.14	0.59 ± 0.10	0.58 ± 0.28	67 ± 6	14 ± 3	19 ± 8	85 ± 6

SH-SY5Y cells were incubated in the absence or presence of 1 m*M* Oxo-M for 30 min and then subjected to hypotonic lysis and differential centrifugation. Cell lysates, P_1, V_1, and S_2 fractions were then assayed for enzyme activity. Results shown are mean ± SEM values for three to five separate experiments. Relative specific activity = activity of enzyme in a subcellular fraction/activity of enzyme in whole cell lysate.

(177 ± 35 and 201 ± 60% of control, respectively, n = 4, $p < 0.05$) and a 14–15% decrease in P_1 immunoreactivity. In contrast, no reduction in immunoreactivity of $G_{\alpha q}$ and $G_{\alpha 11}$ was detected in whole cell lysates obtained from cells incubated with Oxo-M. In addition, no increase in $G_{\alpha s}$ immunoreactivity in the V_1 fraction was observed after agonist pretreatment. To further evaluate the specificity of Oxo-M–induced changes in $G_{\alpha q/\alpha 11}$ and $G_{\alpha s}$, intact cells were incubated with Oxo-M for 24–48 h. Under these conditions, immunoreactivity of $G_{\alpha q}$ and $G_{\alpha 11}$ in whole cell lysates was reduced by 71 ± 16 and 49 ± 16%, respectively (n = 4). In contrast, a significantly smaller decrease in $G_{\alpha s}$ immunoreactivity (26 ± 7%) was observed (Fig. 2B). The distribution of G_β was also determined in the subcellular fractions. Although the P_1 fraction was the most enriched compared with cell lysates (166 ± 8%, n = 5), significant immunoreactivity was also associated with the V_1 fraction (68 ± 3%, n = 5). No increase in G_β immunoreactivity in the V_1 fraction was observed after exposure of the cells to Oxo-M for 30 min.

Subcellular distribution of PLC isozymes

PLC present in intact cells and subcellular fractions was determined either by means of measurement of the release of labeled inositol phosphates or after western blot analysis and quantitative densitometry. When assessed using exogenously added [^{3}H]PIP as substrate, most of the PLC activity (76–77%) was recovered in the S_2 fraction with much less activity localized to the P_1 fraction (19%). Five percent or less of PLC activity was recovered in the V_1 fraction (relative sp. act. 0.29–0.42; Table 2). No significant changes in the activity or distribution of PLC occurred after Oxo-M pretreatment. Because individual PLC isozymes may be differentially localized within the cell, the subcellular distributions of PLC-β1 (primarily α_q-linked), PLC-β3 (α_q and $\beta\gamma$-linked), and PLC-γ1 (linked to receptor tyrosine kinases) were evaluated by western blot analysis (Fig. 3). PLC-β1 was enriched in the P_1 fraction (180 ± 22% of that present in cell lysate, n = 4) but not in either the V_1 or the S_2 fractions (50 ± 4 and 11 ± 3%, respectively, n = 3). PLC-β3 was significantly more enriched in the V_1 fraction (210 ± 31% of that present in cell lysates, n = 5) than in the P_1 fraction (125 ± 11%, n = 5), whereas much less immunoreactivity was observed in the S_2 fraction (27 ± 2%, n = 5). In contrast, PLC-γ1 was most enriched in the S_2 fraction (677 ± 64% of cell lysate, n = 3). PLC-γ1 immunoreactivity in the V_1 fraction equaled that present in the cell lysate (120 ± 35%, n = 3) but was virtually absent from the P_1 fraction (10

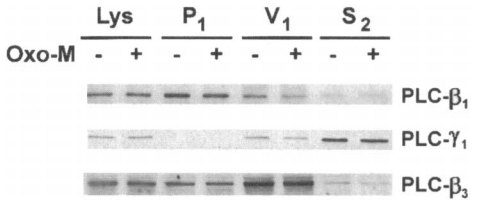

FIG. 3. Western blot analysis of PLC-β1, PLC-β3, and PLC-γ1 in subcellular fractions derived from control or Oxo-M–pretreated cells. Experimental conditions were as described in the legend to Fig. 2A and antibodies to PLC-β1, PLC-β3, and PLC-γ1 were used at final concentrations of 0.25, 0.1, and 0.1 μg/ml, respectively. Results shown are representative of those obtained in three or four separate experiments.

± 5% of cell lysate, n = 4). No statistically significant changes in the subcellular distribution of either PLC-β1 or γ1 were detected in response to Oxo-M pretreatment of cells, whereas a small but statistically significant reduction (14%) in the distribution of PLC-β3 was observed for the V_1 fraction (Fig. 3).

DAG availability, PA formation, and metabolism

After the activation of PLC-linked receptors in intact cells, increases in DAG availability and PA formation are consistently observed. To determine whether these changes are also evident in the cellular compartment enriched in endocytosed mAChRs, DAG mass and PA formation were monitored in subcellular fractions obtained from both control and Oxo-M–treated cells. The concentration of DAG in the V_1 fraction obtained from control cells was relatively low (24% of that present in the P_1 fraction, see Table 3). When the same V_1 fraction was incubated in an "intracellular-like" KGEH buffer that contained [^{32}P]ATP, the [^{32}P]PA labeling observed was also <20% of that obtained for the P_1 fraction (Fig. 4A). The relatively limited synthesis of [^{32}P]PA in the V_1 fraction can be attributed to the restricted availability of DAG, rather than a low activity of DAG kinase, because both P_1 and V_1 fractions possessed similar activities of this enzyme (Table 2). To determine the molecular species of PA formed (and hence, the source of DAG backbone), the [^{32}P]PA was methylated and subjected to argentation TLC (Van Rooijen et al., 1985; see Fig. 5). Under basal conditions, much of the [^{32}P]PA label (in both V_1 and P_1 fractions) was present in the mono- and dienoic forms, whereas <20% was recovered in the tetraenoic species (indicative of an inositide origin). When the cells were exposed to Oxo-M, significant increases in the concentration of DAG were observed in intact cells and P_1 and V_1 fractions (Table 3). The attendant increase in [^{32}P]PA labeling, which was most evident for the V_1 fraction (212 ± 20% of control, n = 11), reflected this increased availability of DAG (Fig. 4B). Analysis of the newly formed [^{32}P]PA indicated that approximately half was of the mono- and dienoic species, with the remainder recovered as either the tetra- or pentaenoic species. Increases in the labeling of the tetraenoic species of PA were significantly greater in the V_1 fraction (680 ± 29%) than in the P_1 fraction (385 ± 26% of control, $p < 0.05$). In contrast, no significant differences in the labeling of the other molecular species of PA in the P_1 and V_1 fractions were observed (Fig. 5). The V_1 fraction also possesses the potential for the further conversion of PA into CDP-DAG, because CDP-DAG synthase activity was detected in this fraction (Table 2).

To determine whether the agonist-mediated increase in DAG accumulation and PA formation reflected the activation of mAChRs that had been endocytosed into the V_1 fraction, cells were made hypertonic (600 mOsm) by the addition of sucrose, a condition under which the agonist-induced translocation of mAChRs is essentially abolished (Fig. 6; see Slowiejko et al., 1996). However, pretreatment of cells with Oxo-M under hypertonic conditions still resulted in robust increases in [^{32}P]PA labeling (Fig. 6). Further evidence to indicate that the receptor-mediated changes in DAG

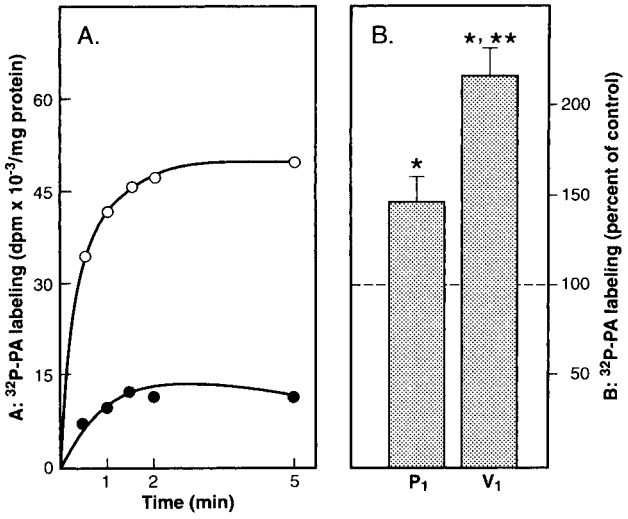

FIG. 4. [^{32}P]PA labeling in subcellular fractions derived from control or Oxo-M–pretreated cells. SH-SY5Y cells were preincubated with either buffer A or 1 m*M* Oxo-M for 30 min before cell lysis and fractionation. **A:** Either V_1 (●) or P_1 (○) fractions from control cells were incubated in KGEH buffer and [^{32}P]ATP for the times indicated. Reactions were terminated by the addition of $CHCl_3/CH_3OH$ (1:2) and lipids extracted and separated as described in Materials and Methods. Results shown are representative of those obtained in three separate experiments and are mean values of triplicate replicates. **B:** The increases in [^{32}P]PA labeling observed in P_1 and V_1 fractions after the Oxo-M pretreatment were monitored (2-min incubation period). Values are expressed as PA labeling (percentage) relative to control cells (buffer A pretreatment). Results shown are mean ± SEM values for 11 separate experiments. *Different from control, $p < 0.05$; **different from the P_1 values, $p < 0.05$. In some studies, the concentration of ATP was increased from 50 to 200 μ*M*, with no change in relative labeling patterns (see Materials and Methods).

TABLE 3. *DAG concentrations in intact cells and subcellular fractions derived from control and Oxo-M–stimulated cells*

	DAG mass (nmol/mg of protein)		
	Intact cells	P_1	V_1
Control	2.12 ± 0.40	5.58 ± 0.45	1.35 ± 0.12
Oxo-M	3.31 ± 0.60[a]	8.81 ± 0.88[a]	2.39 ± 0.34[a]
Percentage of control	161 ± 15	160 ± 13	176 ± 11

SH-SY5Y cells were incubated for 30 min in the absence or presence of 1 m*M* Oxo-M. Cells were then lysed and subjected to differential centrifugation. Aliquots of either intact cells or P_1 or V_1 fractions were then assayed for DAG mass, as described in Materials and Methods. Results shown are means ± SEM for four separate experiments.

[a] Different from control, $p < 0.05$.

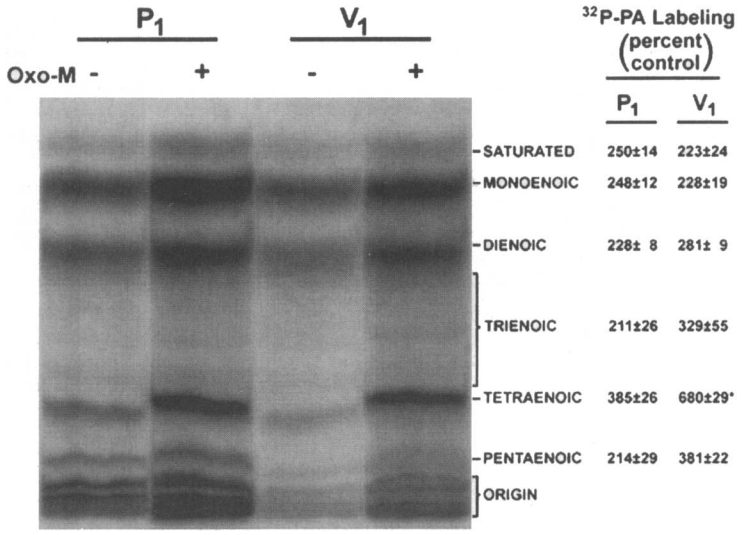

FIG. 5. Species of [^{32}P]PA labeled in P_1 or V_1 fractions obtained from control or Oxo-M–pretreated cells. Cells were incubated for 30 min at 37°C in either the absence (−) or presence (+) of 1 m*M* Oxo-M. Cells were then lysed and subcellular fractions prepared. P_1 and V_1 fractions were incubated with [^{32}P]-ATP and the labeled PA isolated and extracted. PA was then methylated with diazomethane and subjected to argentation TLC (see Materials and Methods). The autoradiographic pattern of the species of [^{32}P]PA labeled is shown and is representative of three experiments performed. Results are expressed as ^{32}P labeling of each species of PA observed after agonist pretreatment (percentage of controls; mean ± SEM, n = 3). *Different from corresponding value in P_1 fraction.

concentration and PA formation do not result from activation of mAChRs within the V_1 fraction was obtained from determination of the kinetics of the two responses. Whereas the $t_{1/2}$ for mAChR translocation is ~12 min (Fig. 1A), the corresponding value for the increase of [^{32}P]PA labeling is 2–3 min (data not shown). Thus, the signaling response in the V_1 fraction (as monitored by the formation of [^{32}P]PA) occurs before and independently of the endocytosis of the mAChR.

Inositol lipid substrate availability

Whether the availability of inositol lipid substrate is a factor that limits the ability of endocytosed mAChRs to activate PLC was assessed by ^{32}P-metabolic labeling studies and by direct measurement of the relevant enzyme activities. When P_1 and V_1 fractions obtained from control SH-SY5Y cells were incubated in KGEH buffer with [^{32}P]ATP, >70% of the radiolabel present in the lipid fraction was recovered in PIP_2 and PIP at all incubation times. However, pronounced differences in the ^{32}P-labeling of the polyphosphoinositides were observed for the two subcellular fractions. In particular, PIP_2 was poorly labeled in the V_1 fraction (16 ± 2% of that observed for the P_1 fraction at 2 min, n = 6, $p < 0.05$; Fig. 7A). In contrast, [^{32}P]PIP labeling in the V_1 fraction exceeded that observed for the P_1 fraction (139 ± 13% at 2 min, n = 6; Fig. 7B). Prior exposure of the cells to Oxo-M resulted in a significant reduction in the subsequent ^{32}P-labeling of both PIP and PIP_2 in the P_1 fraction (12–30%) but was without effect on ^{32}P-polyphosphoinositide labeling in the V_1 fraction (Fig. 7C). Consistent with the observations made from the metabolic labeling studies, there were pronounced differences in the subcellular distribution of three key enzymes of inositol lipid synthesis, namely, PI synthase, PI kinase, and PIP kinase (Table 2). Neither PI synthase nor PIP kinase was enriched in the V_1 fraction (relative sp. act. ~0.8 and 0.50, respectively), both of these enzymes being recovered predominantly in the P_1 fraction. In marked contrast, 37–39% of PI kinase activity recovered was present in the V_1 fraction (relative sp. act. ~2.2), an observation consistent with the enhanced ^{32}P-labeling of PIP in this

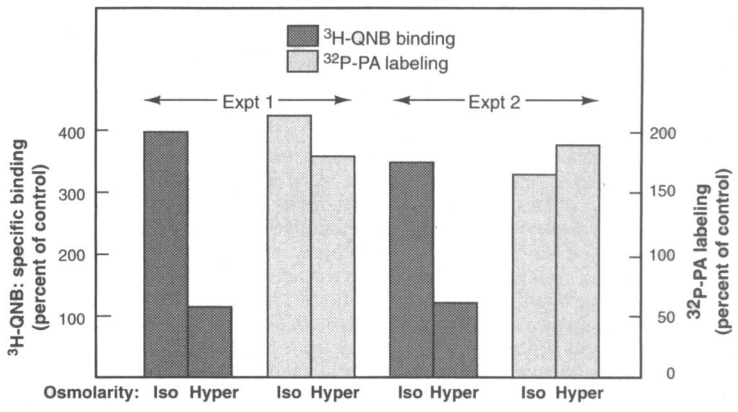

FIG. 6. Agonist-induced mAChR translocation and increased [^{32}P]PA labeling are distinct events. SH-SY5Y cells were incubated under either isotonic (325 mOsm:Iso) or hypertonic (600 mOsm:Hyper) conditions in either the absence or presence of 1 m*M* Oxo-M before cell lysis and fractionation. V_1 fractions were monitored for either [^{3}H]QNB binding or [^{32}P]PA labeling. Results are expressed as either [^{3}H]QNB binding or [^{32}P]PA labeling (percentage of controls).

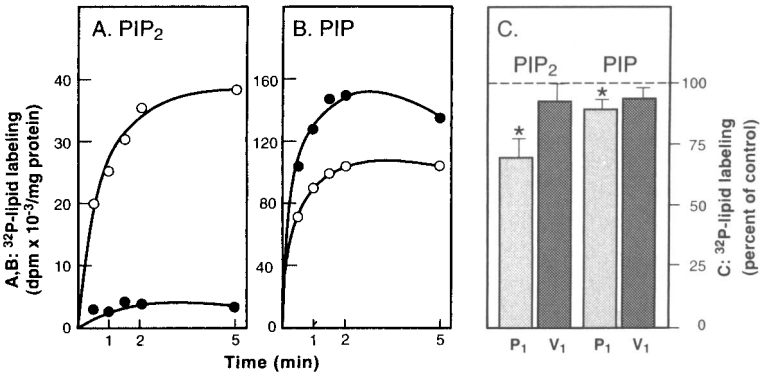

FIG. 7. Polyphosphoinositide labeling in subcellular fractions derived from control or Oxo-M–pretreated cells. SH-SY5Y cells were preincubated with either buffer A or 1 m*M* Oxo-M for 30 min before cell lysis and fractionation. **A** and **B:** Either V_1 (●) or P_1 (○) fractions from control cells were incubated in KGEH buffer and [^{32}P]ATP for the times indicated. Reactions were terminated by the addition of $CHCl_3/CH_3OH$ (1:2) and lipids extracted and separated as described in Materials and Methods. Results shown are representative of three separate experiments, and are mean values of triplicate replicates. **C:** The decreases in [^{32}P]PIP_2 and [^{32}P]-PIP observed in P_1 and V_1 fractions after Oxo-M pretreatment were monitored (2-min incubation period). Values are expressed as [^{32}P]PIP or [^{32}P]PIP_2 labeling observed after agonist pretreatment (percentage of controls; mean ± SEM, n = 9). *Different from control, $p < 0.05$. In some experiments, the concentration of ATP used in the assays was increased from 50 to 200 μM, without any difference in the relative labeling of individual lipids (see Materials and Methods).

fraction. No changes in the amount or distribution of enzyme activities were observed after the pretreatment of cells with Oxo-M (Table 2).

DISCUSSION

In the present study, evaluation of a possible functional role for internalized mAChRs in SH-SY5Y cells has been facilitated by the isolation of a subcellular fraction (V_1) that is enriched in endocytosed receptors. Previously, a "light vesicle" fraction obtained after density gradient centrifugation of whole cell lysates has been used to monitor the subcellular localization of internalized cell surface receptors (Waldo et al., 1983; Harden et al., 1985; Lohse et al., 1990; Sillence and Downes, 1993; Svoboda and Milligan, 1994). Although both the V_1 and light vesicle fractions are heterogeneous, the use of a differential centrifugation procedure to obtain V_1 results in the isolation of an endocytic fraction that is recovered more rapidly and in better yield. Moreover, the characteristics of the V_1 fraction and light vesicle fractions are similar. For example, the V_1 fraction (1) demonstrates a comparable degree of enrichment of mAChRs (four- to fivefold) upon agonist addition, (2) is enriched in smooth endoplasmic reticulum, (3) is essentially devoid of plasma membrane, and (4) exhibits similar equilibrium density characteristics as the light vesicular fraction when applied to sucrose density gradients. In addition, radioligand binding studies indicate that mAChRs in the V_1 fraction are relatively inaccessible to hydrophilic ligands, which suggests that receptors are present in endocytic "inside-out" vesicles. Although fewer mAChRs are recovered in the V_1 fraction than are lost from the cell surface of intact cells, the characteristics of mAChR translocation into this subcellular fraction resemble those observed for receptor sequestration in intact cells in terms of kinetics and dependence on agonist concentration and efficacy.

For internalized mAChRs to activate PLC, both G_q and PLC must be present in the same endosomal compartment. Although relatively little $G_{\alpha q}$ and $G_{\alpha 11}$ are present in the V_1 fraction obtained from quiescent cells (~2–3% of total), this value increases to 4–6% upon exposure of the cells to Oxo-M, whereas no statistically significant increase in immunoreactivity associated with either $G_{\alpha s}$ or G_β (of which ~14% of the cellular complement is localized to V_1) was observed for the same fraction. A similar agonist-induced translocation of the α subunits of G_q and G_{11} has previously been reported for Chinese hamster ovary cells transfected with the cDNA for the m_1 mAChR (Svoboda and Milligan, 1994), although in the latter study an agonist-induced translocation of $G_{\alpha s}$ was also observed. The endocytosis of $G_{\alpha q}$ and $G_{\alpha 11}$ in SH-SY5Y cells in response to Oxo-M addition could indicate either a functional role for the G protein in the sustained coupling of internalized mAChRs to PLC or, alternatively, the intracellular targeting of these subunits before lysosomal degradation. Consistent with the latter possibility is the observation that $G_{\alpha q}$, $G_{\alpha 11}$, and mAChRs all undergo down-regulation in response to chronic mAChR activation, whereas $G_{\alpha s}$ is much less affected (Fig. 2B). The reductions in $G_{\alpha q}/G_{\alpha 11}$ immunoreactivity (49–71%) were similar in magnitude to the loss of mAChRs (52%; see legend to Fig. 2). This observation is consistent with previous studies that indicate that both PLC-coupled wild-type receptors and G_q undergo down-regulation at approximately the same rate (Milligan et al., 1996). Relatively little functional PLC activity (~5% of total) was recovered in the V_1 fraction and western blot analysis confirmed the relative deficiency of the isoform of PLC ($\beta 1$), which is considered to be primarily linked to $G_{\alpha q}/G_{\alpha 11}$. Unexpectedly, however, appreciable PLC-$\beta 3$ and PLC-$\gamma 1$ immunoreactivity was found to be associated with the V_1 fraction. Although the significance of the latter finding is presently unclear, the results could suggest that PLC functional activity is highly regulated in this fraction. In contrast to the results obtained for $G_{\alpha q}$ and $G_{\alpha 11}$, no

translocation of any of these three isoforms of PLC into the V_1 fraction was observed after agonist addition.

Although neither $G_{\alpha q}/G_{\alpha 11}$ nor PLC-β1 is abundant in V_1, substantial increases in DAG mass and [^{32}P]PA labeling are observed in this fraction after agonist pretreatment of cells. Moreover, a significant proportion of the newly formed [^{32}P]PA is of a tetraenoic species, suggesting that the DAG backbone is derived from an inositol lipid. Although these data could be interpreted as evidence in favor of the ability of internalized mAChRs to continue to hydrolyze phosphoinositides, two lines of evidence challenge a functional role for sequestered mAChRs. First, the increases in [^{32}P]PA labeling persist under hypertonic conditions in which mAChR translocation is prevented (see Fig. 6). Second, increases in [^{32}P]PA labeling ($t_{1/2}$ = 2 min) occur *before* the endocytosis of the mAChR ($t_{1/2}$ = 12 min). Thus, it is more likely that DAG formation occurs primarily at a site other than the V_1 fraction (presumably the plasma membrane) and that a redistribution of at least some of this lipid to the endosomal compartment (and its phosphorylation to PA) occurs thereafter. Although increases in phosphoinositide hydrolysis may occur primarily at subcellular location(s) other than the V_1 fraction, the products of phosphoinositide turnover may nonetheless play an important role in the intracellular trafficking of mAChRs. For example, increases in DAG concentration have been demonstrated to promote membrane fusion (Siegel et al., 1989; Sánchez-Migallón et al., 1994). Furthermore, the formation of PIP has also been linked to membrane fusion and intracellular trafficking events (Del Vecchio and Pilch, 1991).

We have previously calculated that for continuous mAChR-stimulated phosphoinositide hydrolysis to occur in SH-SY5Y cells, the entire PIP_2 pool must be replenished every 45–60 s (Fisher et al., 1994). This prompted an examination of the ability of the V_1 fraction to synthesize phosphoinositides. Although all of the enzymes necessary for the breakdown and resynthesis of inositol lipids can be detected, the most pronounced observation was the relative inability of the V_1 fraction to synthesize PIP_2, as was apparent from both ^{32}P-labeling studies and direct enzyme measurement. Because PIP formation occurs readily in this fraction, precursor availability is unlikely to be a rate-limiting factor in PIP_2 synthesis. In addition, because PIP kinase activity in SH-SY5Y cells is recovered predominantly in the particulate fractions (as previously observed for C_6 glioma cells; see Morris et al., 1990), it is unlikely that, in the intact cell, the cytosol would provide a significant source of the enzyme. Accordingly, it is conceivable that the limited ability of the V_1 fraction to synthesize PIP_2 may represent one of the key factors that prevents the sustained coupling of endocytosed mAChRs to PLC. The low activities of both PIP and DAG kinases in the V_1 fraction, along with the enrichment of PI kinase, is strikingly reminiscent of the distribution of these same enzymes in glucose transporter–containing vesicles and clathrin-coated vesicles (Campbell et al., 1985; Del Vecchio and Pilch, 1991) and raises the possibility that this enzyme profile may be functionally relevant in structures dedicated to intracellular trafficking.

In summary, although all components necessary for operation of the phosphoinositide cycle can be detected in the endocytic fraction into which mAChRs are endocytosed, the major conclusion to emanate from the present study is that once internalized, mAChRs appear to possess little, if any, potential to further activate PLC. One factor that may explain the refractoriness of internalized receptors to activate PLC is the very limited capacity of this endocytic fraction to synthesize the primary lipid substrate PIP_2.

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