Arch Pharm Res Vol 28, No 11, 1275-1281, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Direct Involvement of G Protein $\alpha_{q/11}$ Subunit in Regulation of Muscarinic Receptor-Mediated sAPPα Release

Jin Hyoung Kim[1] and Hwa-Jung Kim

Research Institute of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea and [1]Division of Pharmaceutical Bioscience, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Korea

(Received July 27, 2005)

The $G_{q/11}$ protein-coupled receptors, such as muscarinic (M1 & M3) receptors, have been shown to regulate the release of a soluble amyloid precursor protein (sAPPα) produced from α-secretase processing. However, there is no direct evidence for the precise characteristics of G proteins, and the signaling mechanism for the regulation of $G_{q/11}$ protein-coupled receptor-mediated sAPPα release is not clearly understood. This study examined whether the muscarinic receptor-mediated release of sAPPα is directly regulated by $G\alpha_{q/11}$ proteins. The HEK293 cells were transiently cotransfected with muscarinic M3 receptors and a dominant-negative minigene construct of the G protein α subunit. The sAPPα release in the media was measured using an antibody specific for sAPP. The sAPPα release enhancement induced by muscarinic receptor stimulation was decreased by a $G_{q/11}$ minigene construct, whereas it was not blocked by a control minigene construct (the Gα carboxy peptide in random order, $G\alpha_qR$) or $G\alpha_i$ constructs. This indicated a direct role of the $G\alpha_{q/11}$ protein in the regulation of muscarinic M3 receptor-mediated sAPPα release. We also investigated whether the transactivation of the epidermal growth factor receptor (EGFR) by a muscarinic agonist could regulate the sAPPα release in SH-SY5Y cells. Pretreatment of a specific EGFR kinase inhibitor, tyrophostin AG1478 (250 nM), blocked the EGF-stimulated sAPPα release, but did not block the oxoM-stimulated sAPPα release. This demonstrated that the transactivation of the EGFR by muscarinic receptor activation was not involved in the muscarinic receptor-mediated sAPPα release.

Key words: sAPPα Release, Muscarinic receptor, G protein, Epidermal growth factor receptor, SH-SY5Y cells

INTRODUCTION

G protein-coupled receptors (GPCRs), which link extracellular stimuli including light, odorants, hormones, and neurotransmitters to effector molecules, represent a major mechanism for signal transduction in mammalian cells. It is currently estimated that as much as 1% of the mammalian genome codes for GPCR (Morris *et al.*, 1999). However, the exact mechanisms involved in the signal transduction events that follow activation of many GPCR are not fully understood. It has been known that the release of soluble amyloid precursor protein (sAPPα) formed by α-secretase is regulated by the activation of the phospholipase C (PLC)-linked $G\alpha_{q/11}$-coupled receptors. These receptors include muscarinic M1 and M3 receptor subtypes (Nitsch *et al.*, 1992; Hung *et al.*, 1993; Buxbaum *et al.*, 1994; Slack *et al.*, 1995), metabotropic glutamate receptors (Lee *et al.*, 1995; Lee and Wurtman, 1997; Nitsch *et al.*, 1997; Jolly-Tornetta *et al.*, 1998), and serotonergic receptors (Nitsch *et al.*, 1996), but not by activation of adenylyl cyclase-linked GPCRs including muscarinic M2 and M4 receptors (Nitsch *et al.*, 1992). However, whether the G-protein α subunit (Gα) directly regulates the sAPPα release has not been examined.

Previous studies have implicated the carboxyl terminus of G protein α subunits in mediating receptor-G protein interaction and selectivity (Sprang, 1997; Bourne, 1997). Carboxyl termini from G protein α subunits are important sites of receptor binding. Additionally, peptides corresponding to the carboxyl terminus can be used as competitive inhibitors of receptor-G protein interactions (Hamm *et al.*,

Correspondence to: Hwa-Jung Kim, College of Pharmacy, Ewha Womans University, 11-1, Daehyun-Dong, Seodaemun-Gu, Seoul, 120-750, Korea
Tel: 82-2-3277-3021, Fax: 82-2-3277-3051
E-mail: hjkim@ewha.ac.kr

1988; Martin *et al.,* 1996; Resenick *et al.,* 1994). This interaction is quite specific as Gilchrist *et al.* (1999) ascertained that a difference in one amino acid can abolish the ability of the $G\alpha_{i1/2}$ peptide to bind the A1 adenosine receptor-G protein interface. In the present study, we used different Gα carboxy-terminal peptides to investigate whether the activation of the Gα subunit protein is directly involved in the regulation of sAPPα release mediated by muscarinic receptors.

It was recognized that GPCRs, including members of the muscarinic acetylcholine receptor family, are functionally linked to the mitogen-activated protein kinase (MAPK) cascade *via* several distinct pathways. Typically, activation of MAPK by GPCRs involves tyrosine phosphorylation of one or more proteins acting as scaffolds, forming complexes with Grb2–mSos. This promotes the activation of the Ras-MAPK cascade (Della Rocca *et al.,* 1999). One such scaffolding protein is the epidermal growth factor (EGF) receptor. EGF-independent activation of this receptor by GPCRs, a phenomenon referred to as 'transactivation' (Luttrell *et al.,* 1997; Daub *et al.,* 1996, 1997; Cunnick *et al.,* 1998; Eguchi *et al.,* 1998; Keely *et al.,* 1998; Li *et al.,* 1998), results in the formation of a complex composed of the phosphorylated EGF receptor and adapter proteins Shc and Grb2 (Daub *et al.,* 1996; Eguchi *et al.,* 1998; Keely *et al.,* 1998). The adapter proteins mediate the recruitment of mSos to the complex. This recapitulates the sequence of events set into motion by the direct activation of growth factors by their ligands. It was shown that the muscarinic agonist transactivated the EGF receptor in T_{84} cells by a mechanism involving elevation in intracellular calcium, activation of the soluble tyrosine kinase Pyk-2, recruitment of $p60^{src}$ kinase, and subsequent phosphorylation and activation of the EGF receptor (Keely *et al.,* 2000). In this study, we examined whether the transactivation of EGF receptors by the muscarinic agonist could regulate the sAPPα release in SH-SY5Y cells.

MATERIALS AND METHODS

Mammalian cell culture

SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) and HEK-293 cells (ATCC CRL-1573) were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37°C in humidified conditions under 5% CO_2. For the experiments, SH-SY5Y cells were plated in 6 well plates and HEK293 cells were plated in 12 well dishes (4×10^5 cells/well) coated with poly-L-lysine (0.01 mg/mL).

Measurement of sAPPα release

The measurement of sAPPα release was performed as described by Nitsch *et al.* (1992) with modifications. Cells were cultured to confluency in multiwell dishes (6-well format). Prior to the addition of the drug, cells were washed with serum-free media and pre-treated with various agents in serum-free media for 30 min following a treatment of oxoM in serum-free media for 1 h. The conditioned medium from each well was centrifuged to remove debris, then desalted, dried, and reconstituted in a SDS loading buffer. Total cell protein per dish was measured with the bicinchoninic acid assay (Pierce). Reconstituted culture media protein extracts with similar amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis on 10% gels, and transferred to polyvinylidene fluoride membranes. These were immunoblotted using the anti-preA4 APP monoclonal antibody (clone 22C11, 1:125, Roche Molecular Biochemicals) and bands were visualized using a chemiluminescence method (Amersham Pharmacia Biotech). Data collection and processing were performed with the luminescent image analyzer LAS-1000 and IMAGE GAUSE software (Fuji Film, Japan).

Construction of Gα carboxyl-terminal minigenes

The Gα minigenes ($G\alpha_{11}$ and $G\alpha_s$) were provided by Dr. Heidi Hamm (Northwestern University, U.S.A.). Other minigenes were constructed as described by Gilchrist *et al.* (1999). The cDNA encoding for the last 11 amino acids of human $G\alpha_i$ and $G\alpha_q$ subunits or the $G\alpha_q$ carboxy terminus in random order ($G\alpha_qR$) were synthesized with newly engineered 5'- and 3'-ends (Fig. 2A). The 5'-end contained a *Hind*III site followed by the ribosome binding consensus sequence (5'-GCCGCCACC-3'), a methionine (ATG) for translation initiation, and a glycine (GGA) to protect the ribosome binding site during translation. Also protected by the glylcine was the nascent peptide against proteolytic degradation, while the A *BamH*I site was synthesized at the 3'-end immediately following the translation stop codon (TGA). The complimentary DNA were annealed in a buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol (New England Biolabs) at 85°C for 15 min, and then slowly cooled to room temperature. The annealed cDNA were ligated at room temperature for 90 min into pcDNA 3.1(+) plasmid vector (Invitrogen), previously cut with *Hind*III and *BamH*I. To verify that an insert was present, the plasmid DNA was digested with *Nco*I at 37°C for 1 h and run on a 1.5% agarose gel. The vector alone produced 3 bands (3.3, 1.4, and 0.7 kilobases), whereas the vector with insert resulted in 4 bands (3.3, 1.0, 0.7, and 0.4 kilobases).

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine minigene RNA expression, transiently transfected HEK 293 cells were twice washed with

phosphate-buffered saline and total RNA was processed according to the manufacturer's protocol (Clontech). cDNA was made from total RNA using a RT-PCR (Promega) according to the manufacturer's protocol. To verify the presence of an insert in cells transfected with Gá minigene constructs, their cDNA was used as a template for PCR with forward and reverse primers corresponding to the Gα insert and vector, respectively (forward: 5'-GCTTGCCGCCACCATGGGA; reverse: 5'-CCGCCGCGCTTAATGCGCC).

Transient transfection of HEK293 cells

The day before transfection, the HEK 293 cells were trypsinized, counted, and plated in 12-well plates at 4×10^5 cells per well, so that they were 90-95% confluent at the moment of transfection. For each well of cells to be transfected, DNAs were diluted into 100 μL of serum- and antibiotics-free DMEM. Additionally, 4 μL of LIPOFECTAMINE 2000 (LF2000™) reagent (GIBCO) were diluted into 100 μL of serum- and antibiotics-free DMEM. The diluted DNA along with the diluted LF 2000 were combined within 5 min and incubated at room temperature for 20 min to allow DNA-LF2000 Reagent complexes to form. Then they were directly added to each well and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO_2 incubator for 48 h.

RESULTS AND DISCUSSION

Muscarinic receptor-mediated increase in sAPPα release in SH-SY5Y cells

It was previously shown that the activations of muscarinic receptors (M1 and M3 subtypes) increased sAPPα release (Nitsch *et al.*, 1992; Slack *et al.*, 1993). Similarly, we observed that the activation of muscarinic receptors in SH-SY5Y cells by exposure to a muscarinic agonist, oxoM (1 mM), led to a significant increase in sAPPα release into the culture medium in a time-dependent manner. This oxoM-induced sAPPα release was blocked by pre-treatment with a muscarinic antagonist, atropine (Fig. 1).

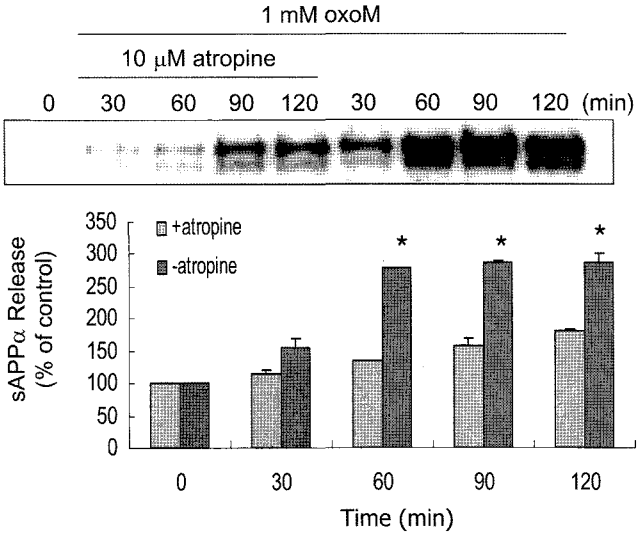

Fig. 1. Muscarinic receptor-mediated increase of sAPPα release in SH-SY5Y cells. Cells were pretreated with vehicle and atropine (10 μM), and then stimulated with 1 mM oxoM. The secreted sAPPα was measured as described in "*Materials and Methods*". Data are the means ± S.E.M. of three experiments. Significance values indicate differences compared with oxoM-treated cells in the absence of atropine. * $p < 0.05$.

Construction of Gα cDNA minigenes and determination of minigene RNA expression

Carboxyl termini from G protein α subunits are known to be important sites of direct interaction with GPCRs. The peptides corresponding to the carboxyl terminus can be used as competitive inhibitors of receptor-G protein interactions (Hamm *et al.*, 1988; Martin *et al.*, 1996; Resenick *et al.*, 1994). The inhibitory effects of the minigene constructs were confirmed by Gilchrist *et al.* (1999), by showing that $G\alpha_i$ minigene constructs, encoding the last 11 amino acids of the carboxyl terminus of $G\alpha_{i1/2}$, resulted in an 85% inhibition of the M2 muscarinic receptor-activated inwardly rectifying the K^+ channel. In the present study, we used the different Gα carboxy-terminal peptides to investigate whether the activation of Gα subunit protein is directly involved in the regulation of sAPPα release mediated by muscarinic receptors.

Table I shows the alignment of the last 11 amino acid residues from human Gα subunits. The cDNA encoding the last 11 amino acids of human Gα subunits ($G\alpha_i$, $G\alpha_q$) or the $G\alpha_q$ carboxy terminus in random order ($G\alpha_qR$) were

Table I. Alignment of the last 11 amino acid residues from human Gα subunits

Gi1/2	I	K	N	N	L	K	D	C	G	L	F
Gi3	I	K	N	N	L	K	E	C	G	L	Y
Gq/11	L	Q	L	N	L	K	E	Y	N	L	V
Gs	Q	R	M	H	L	R	Q	Y	E	L	L
Gt	I	K	E	N	L	K	D	C	G	L	F
Go1	I	A	N	N	L	R	G	C	G	L	Y
Go2	I	A	K	N	L	R	G	C	G	L	Y
Golf	Q	R	M	H	L	K	Q	Y	E	L	L
G12	L	Q	E	N	L	K	D	I	M	L	Q
G13	L	H	D	N	L	K	Q	L	M	L	Q
G14	L	Q	L	N	L	R	E	F	N	L	V
G15/16	L	A	R	Y	L	D	E	I	N	L	L
Gz	I	Q	N	N	L	K	Y	I	G	L	C

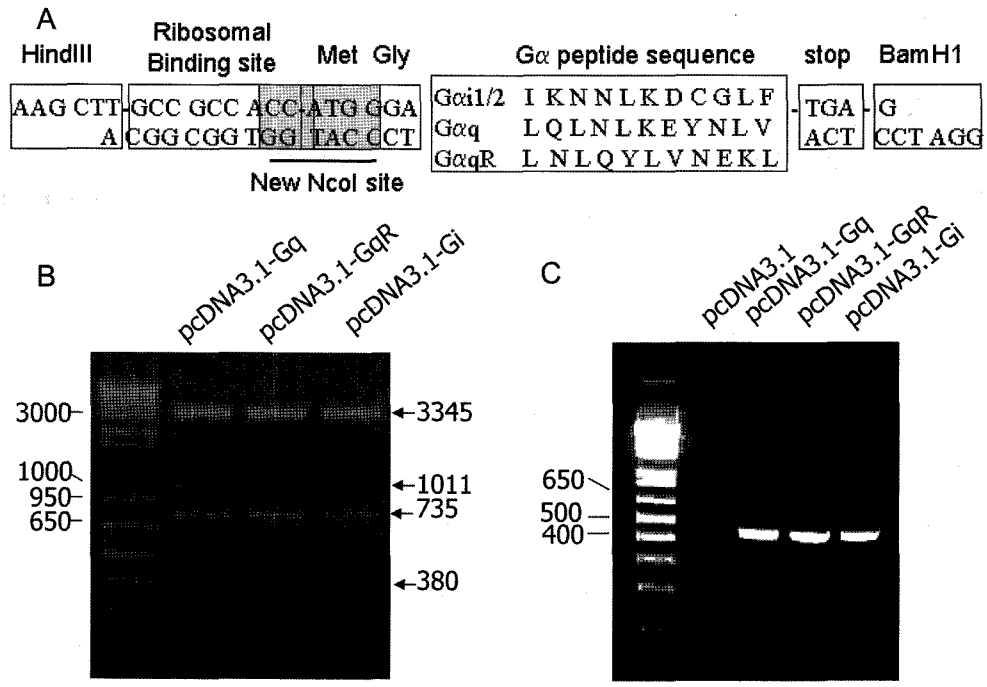

Fig. 2. Construction of Gα cDNA minigenes and RNA expressions of G protein minigenes. (A) All Gα carboxyl-terminal peptide minigenes contain a HindIII restriction enzyme site at the 5'-end followed by a ribosomal binding site sequence,a methionine for translation initiation, a glycine for stabilization of the peptide, the peptide sequence, a stop codon, and a HamH1 restriction enzyme site at the 3'-end. (B) Plasmid DNA was purified, digested with *Nco* I, and separated on a 1.5% agarose gel to determine whether insert was present. Lane 1 is a 1-kilobase pair DNA ladder; lane 2 is pcDNA3.1-$G\alpha_q$; lane 3 is pcDNA3.1-$G\alpha_q$R; and lane 4 is pcDNA3.1-$G\alpha_i$. (C) HEK293 cells were transiently transfected with pcDNA3.1 zeo(+), pcDNA-$G\alpha_q$, pcDNA-$G\alpha_q$R, or pcDNA-$G\alpha_i$, and mRNA was isolated and then RT-PCR analysis was performed. PCR products were separated on 1.5% agarose gels.

constructed as shown in Fig. 2A. To determine whether the insert was present, DNA was cut with *Nco* I and separated on a 1.5% agarose gel. As shown in Fig. 2B, when the insert is present, there is a new *Nco* I site resulting in a shift in the band pattern, such that the digest pattern goes form three bands (3.3, 1.4, and 0.7 kilobases) to four bands (3.3, 1.0, 0.7, and 0.4 kilobases). The presence of the minigene constructs in transfected cells was confirmed by RT-PCR using the cDNA as a template with primers specific for the Gα carboxyl-terminal peptide insert (Fig. 2C).

Regulation of muscarinic receptor-mediated sAPPα release by Gα subunit

There have been several reports indicating the $G\alpha_{q/11}$ protein coupled-receptors including muscarinic receptors (M1, M3), metabotropic glutamate receptors, and bradykinin receptors (Buxbaum *et al.*, 1992, 1994; Hung *et al.*, 1993; Wolf *et al.*, 1995; Lee *et al.*, 1995), regulate sAPPα release. However, whether the Gα protein directly regulates the sAPPα release has not been tested. To get direct evidence of the role of Gα proteins in regulating muscarinic receptor-mediated sAPPα release, we cotransfected $G\alpha_{q/11}$ coupled muscarinic M3 receptors with a different dominant-negative Gα carboxy-terminal peptide minigene construct into HEK 293 cells and tested the effect of the peptide on the sAPPα release enhancement induced by muscarinic receptor agonist. Numerous studies have shown that the carboxyl terminus of the Gα subunits is critical in both mediating receptor-G protein interaction and in receptor selectivity (Gilchrist *et al.*, 1998; Blahos *et al.*, 1998; Onrust *et al.*, 1997). Gilchrist *et al.* (1999) reported that the transfection of different Gα carboxyl-terminal peptides selectively blocked signal transduction through the corresponding G protein. They observed that the $G\alpha_i$ minigene construct completely blocked muscarinic M2 receptor-mediated K^+ channel response, whereas the control minigene construct (empty vector), and $G\alpha_{q/11}$ minigene constructs had no effects on muscarinic M2 receptor-mediated K^+ channel response. The inhibitory effects of the $G\alpha_i$ minigene constructs were specific because the over-expression of peptides corresponding to the carboxyl terminus of $G\alpha_q$ or $G\alpha_s$ had no effect on M2 receptor stimulation of the K^+ channel. By using similar strategies, we observed that oxoM-stimulated sAPPα release was reduced in the $G\alpha_{q/11}$ minigene transfected cells, but was not blocked by control the Gα protein construct (the Gα carboxy peptide in random order, $G\alpha_q$R) or dominant-

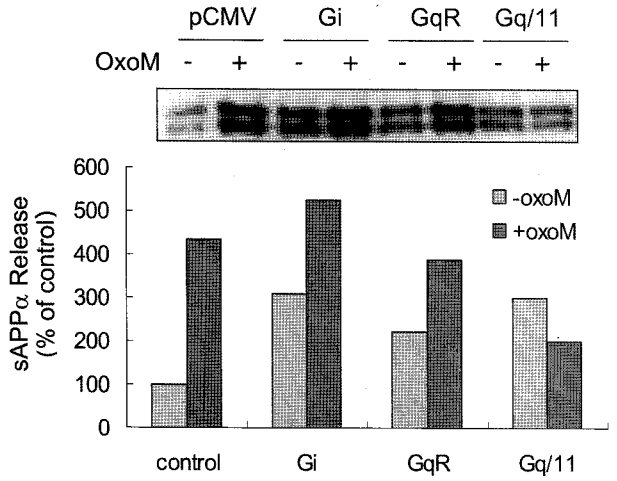

Fig. 3. Effect of dominant-negative Gα carboxy-terminal peptide on sAPPα release induced by muscarinic agonist. HEK293 cells were transiently transfected with M3 plus pcDNA3.1 zeo(+), pcDNA3.1-$G\alpha_i$, pcDNA3.1-$G\alpha_q$R, pcDNA3.1-$G\alpha_q$, or pcDNA3.1-$G\alpha_{11}$. Cells were incubated for 1 h in serum-free medium or in medium containing 1 mM oxoM at 48 h posttrasnfection and the secreted sAPPα was measured as described in "*Materials and Methods*".

negative $G\alpha_i$ (Fig. 3). The transfection of Gα minigene constructs induced a slight increase of the sAPPα release in the absence of oxoM for unknown reasons. This result suggests that $G\alpha_{q/11}$ subunit, especially $G\alpha_q$ and $G\alpha_{11}$, could directly regulate muscarinic receptor-mediated sAPPα release.

Involvement of EGFR cross-talk in muscarinic receptor-mediated sAPPα release

Previous work has shown that muscarinic M1 activation led to the transactivation of the EGF receptors in M1 transfected HEK293 cells (Tasi *et al.*, 1997). Several previous studies have shown that activation of EGF receptors also enhanced the production of sAPPα in non-neuronal cell lines or in neuronal cell lines (Mills *et al.*, 1999; Slack, 2000; Petanceska *et al.*, 1999; De Strooper *et al.*, 2000). To assess the ability of EGF receptor activation to stimulate sAPPα release, the action of EGF on sAPPα release was examined in SH-SY5Y cells. As reported by other studies, EGF receptor activation led to an increase of sAPPα release in SH-SY5Y cells (Fig. 4) (Canet-Aviles *et al.*, 2002; Slack *et al.*, 1997).

Muscarinic receptors have been reported to be coupled with the activation of ERK 1/2 kinase via PKC-dependent and PKC-independent pathways. The latter involving transactivation of an EGF receptor kinase in HEK cells transfected with the M3 receptor subtype (Slack, 2000). To further examine whether EGFR transactivation was involved in the muscarinic receptor-mediated sAPPα release in SH-SY5Y cells, the cells were pretreated with a

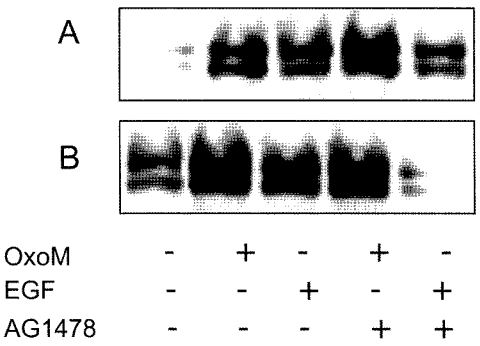

Fig. 4. Effects of AG1478 on oxoM- and EGF-stimulated sAPPα release. Cells were treated with vehicle alone or AG1478 (250 nM) for 30 min and then stimulated with oxoM (1 mM) or EGF (1 mg/mL) for 30 min (A) or 1 h (B) and the secreted sAPPα was measured as described in "*Materials and Methods*".

specific inhibitor of the EGFR kinase, tyrophostin AG1478, for 30 min and then stimulated with oxoM for 30 min or 1 h. Previous reports indicated that tyrophostin AG1478 acts as a specific inhibitor of EGFR kinase activity and has no significant effect on other tyrosine kinase tested (Levitzki *et al.*, 1995). Pretreatment of tyrophostin AG1478 (250 nM) blocked the EGF- stimulated sAPPα release, but did not block the oxoM-stimulated sAPPα release (Fig. 5). These results demonstrates that transactivation of EGF receptor by muscarinic agonist is not involved in muscarinic receptor-mediated sAPPα release.

REFERENCES

Blahos, J. N., Mary, S., Perroy, J., de Colle, C., Brabet, I., Bockaert, J., and Pin, J. P., Extreme C terminus of G protein a-subunits contains a site that discriminates between G_i-coupled metabotropic glutamate receptors. *J. Biol. Chem.*, 273, 25765-25769 (1998).

Bourne, H. R., How receptors talk to trimeric G proteins. *Curr. Opin. Cell Biol.*, 9, 134-142 (1997).

Buxbaum, J. D., Oishi, M., Chen, H. I., Pinkas-Kramarski, R., Jaffe, E. A., Gandy, S. E., and Greenfard, P., Cholinergic agonists and interleukin-1 regulate processing and secretion of the Alzheimer beta/A4 amyloid protein precursor. *Proc. Natl. Acad. Sci. U.S.A.*, 89, 10075-10078 (1992).

Buxbaum, J. D., Ruefli, A. A., Parker, C. A., Cypess, A. M., and Greengard, P., Calcium regulates processing of the Alzheimer amyloid protein precursor in a protein kinase C-independent manner. *Proc. Natl. Acad. Sci. U.S.A.*, 91, 4489-4493 (1994).

Canet-Aviles, R. M., Anderton, M., Hooper, N. M., Turner, A. J., and Vaughan, P. F., Muscarine enhances soluble amyloid precursor protein secretion in human neuroblastoma SH-SY5Y by a pathway dependent on protein kinase C(alpha), src-tyrosine kinase and extracellular signal-regulated kinase

but not phospholipase C. *Brain Res. Mol. Brain Res.,* 102, 62-72 (2002).

Checler, F., Processing of the beta-amyloid precursor protein and its regulation in Alzheimer's disease. *J. Neurochem.,* 65, 1431-1444 (1995).

Cunnick, J. M., Dorsey, J. F., Standley, T., Turkson, J., Kraker, A. J., Fry, D. W., Jove, R., and Wu, J., Role of tyrosine kinase activity of epidermal growth factor receptor in the lysophosphatidic acid-stimulated mitogen-activated protein kinase pathway. *J. Biol. Chem.*, 273, 14468-14475 (1998).

Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A., Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature (London),* 379, 557-560 (1996).

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A., Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.,* 16, 7032-7044 (1997).

De Strooper, B. and Annaert, W., Proteolytic processing and cell biological functions of the amyloid precursor protein. *J. Cell Sci.,* 113, 1857-1870 (2000).

Della Rocca, G. J., Van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Lefkowitz, R. J., Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J. Biol. Chem.,* 272, 19125-19132 (1997).

Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M., Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *J. Biol. Chem.*, 274, 13978-13984 (1999).

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J., A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature (London),* 383, 547-550 (1996).

Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., and Inagami, T., Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.,* 273, 8890-8896 (1998).

Gilchrist, A., Mazzoni, M., Dineen, B., Dice, A., Linden, J., Proctor, W. R., Lupica, C. R., Dunwiddie, T., and Hamm, H. E., Antagonists of the receptor-G protein interface block G_i-coupled signal transduction. *J. Biol. Chem.*, 273, 14912-14919 (1998).

Gilchrist, A., Bünemann, M., Li, A., Hosey, M. M., and Hamm, H. E., A dominant-negative strategy for studying roles of G proteins. *J. Biol. Chem.,* 274, 6610-6616 (1999).

Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hoffmann, K. P., Site of G protein binding to rhodopsin mapped with synthetic peptides from the alpha subunit. *Science,* 241, 832-835 (1988).

Hung, A. Y., Haass, C., Nitsch, R. M., Qiu, W. Q., Citron, M., Wurtman, R. J., Growdon, J. H., and Selkoe, D. J., Activation of protein kinase C inhibits cellular production of the amyloid beta-protein. *J. Biol. Chem.,* 268, 22959-22962 (1993).

Keely, S. J., Uribe, J. M., and Barrett, K. E., Carbachol stimulates transactivation of epidermal growth factor receptor and mitogen-activated protein kinase in T84 cells. Implications for carbachol-stimulated chloride secretion. *J. Biol. Chem.*, 273, 27111-27117 (1998).

Keely, S. J., Calandrella, S. O., and Barrett, K. E., Carbachol-stimulated Transactivation of Epidermal Growth Factor Receptor and Mitogen-activated Protein Kinase in T_{84} Cells Is Mediated by Intracellular Ca^{2+}, PYK-2, and $p60^{src}$ *J. Biol. Chem.,* 275, 12619-12625 (2000).

Lee, R. K. K., Wurtman, R. J., Cox, A. J., and Nitsch, R. M., Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. *Proc. Natl. Acad. Sci. U.S.A.,* 92, 8083-8087 (1995).

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J., Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature (London),* 376, 737-745 (1995).

Levitzki, A. and Gazit, A., Tyrosine kinase inhibition: an approach to drug development. *Science,* 267, 1782-1788 (1995).

Li, X., Lee, J. W., Graves, L. M., and Earp, H. S., Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. *EMBO J.,* 17, 2574–2583 (1998).

Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J., G-betagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J. Biol. Chem.,* 272, 4637-4644 (1997).

Martin, E. L., Rens-Domiano, S., Schatz, P. J., and Hamm, H. E., Potent peptide analogues of a G protein receptor-binding region obtained with a combinatorial library. *J. Biol. Chem.,* 271, 361-366 (1996).

Mills, J. and Reiner, P. B., Regulation of amyloid precursor protein cleavage. *J. Neurochem.,* 72, 443-460 (1999).

Morris, A. J. and Malbon, C. C., Physiological regulation of G protein-linked signaling. *Physiol. Rev.,* 79, 1373-430 (1999).

Nitsch, R. M., Slack, B. E., Wurtman, R. J., and Growdon, J. H., Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science,* 258, 304-307 (1992).

Onrust, R., Herzmark, P., Chi, P., Garcia, P. D., Lichtarge, O., Kingsley, C., and Bourne, H. R., Receptor and bg binding sites in the a subunit of the retinal G protein transducin. *Science,* 275, 381-384 (1997).

Petanceska, S. S. and Gandy, S., The phosphatidylinositol 3-

kinase inhibitor wortmannin alters the metabolism of the Alzheimer's amyloid precursor protein. *J. Neurochem.,* 73, 2316-2320 (1999).

Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M., and Lefkowitz, R. J., Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors. *J. Biol. Chem.,* 276, 23155-23160 (2001).

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A., EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature,* 402, 884-888 (1999).

Resenick, M. M., Watanabe, M., Lazarevic, M. B., Hatta, S., and Hamm, H. E., Synthetic peptides as probes for G protein function. Carboxyl-terminal G alpha s peptides mimic Gs and evoke high affinity agonist binding to beta-adrenergic receptors. *J. Biol. Chem.,* 269, 21519-21525 (1994).

Slack, B. E., Nitsch, R. M., Livneh, E., Kunz, G. M., Breu, J., Eldar, H., and Wurtman, R. J., Regulation by phorbol esters of amyloid precursor protein release from Swiss 3T3 fibroblasts overexpressing protein kinase Ca. *J. Biol. Chem.,* 268, 21097-21101 (1993).

Slack, B. E., Breu, J., Muchnicki, L., and Wurtman, R. J., Rapid stimulation of amyloid precursor protein release by epidermal growth factor: role of protein kinase C. *Biochem. J.,* 327, 245-249 (1997).

Slack, B. E., The m3 muscarinic acetylcholine receptor is coupled to mitogen-activated protein kinase *via* protein kinase C and epidermal growth factor receptor kinase. *Biochem. J.,* 348, 381-387 (2000).

Soltoff, S. P., Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y2 receptor. Phorbol ester or $[Ca^{2+}]i$ elevation can substitute for receptor activation. *J. Biol. Chem.,* 273, 23110–23117 (1998).

Soltoff, S. P., Avraham, H., Avraham, S., and Cantley, L. C., Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J. Biol. Chem.*, 273, 2653–2660 (1998).

Sprang, S. R., G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.,* 66, 639-678 (1997).

Tasi, W., Morielli, A. D., and Peralta, E. G., The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J.,* 16, 4597-4605 (1997).

Villa, A., Santiago, J., Garæia-Silva, S., Ruiz-León, Y., and Pascual, A., Serum is required for release of Alzheimer's amyloid precursor protein in neuroblastoma cells. *Neurochem. Int.,* 41, 261-269 (2002).

Wolf, B. A., Werkin, A. M., Jolly, Y. C., Yasuda, R. P., Wolfe, B. B., Konrad, R. J., Manning, D., Ravi, S., Williamson, J. R., and Lee, V. M.-Y., Muscarinic regulation of Alzheimer's disease amyloid precursor protein secretion and amyloid β-protein production in human NT2N cells. *J. Biol. Chem.,* 270, 4916-4922 (1995).

Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K., and Ullrich, A., Critical Role of Calcium- dependent Epidermal Growth Factor Receptor Transactivation in PC12 cell membrane depolarization and bradykinin signaling, *J. Biol. Chem.,* 272, 24767-24770 (1997).