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Tyrosine Kinases of the Src Family Participate in Signaling to MAP Kinase from both G_q and G_i-Coupled Receptors

Tadashi Igishi and J. Silvio Gutkind[1]
Oral and Pharyngeal Cancer Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-4330

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Src-related kinases have been recently implicated in signaling from G_i-coupled receptors to MAP kinase. Whether Src-like kinases participate in MAP kinase activation by the large family of receptors coupled to G proteins of the G_q family is still unclear. Here, we show that a specific inhibitor for Src-like kinases, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1), and dominant negative mutants of Src suppress MAP kinase activation in COS-7 cells when elicited by either m1 and m2 muscarinic receptors, which are typical G_q and G_i-coupled receptors, respectively. Furthermore, activation of MAP kinase by overexpression of $\beta\gamma$ subunits, but not by stimulation with phorbol esters was also inhibited by the dominant-negative Src. In contrast, a dominant negative Pyk2 had only mild effects on m1 and m2 mediated-MAP kinase activation. We concluded that Src like kinase(s), acting downstream from $\beta\gamma$ dimers, play an important role relaying signals from both G_q and G_i-coupled receptors to MAP kinase.

The primary function of mitogen activated protein kinases (MAP kinases) is to convert extracellular stimuli into intracellular signal which, in turn, control the expression of genes that are essential for many cellular processes, including cell growth and differentiation (1). MAP kinases, $p42^{MAPK}$ and $p44^{MAPK}$, also known as ERK2 and ERK1, respectively, can be rapidly activated upon stimulation of receptors of possessing an intrinsic tyrosine kinase activity or receptors that transduce signals through heterotrimeric guanine trisphosphate-binding proteins (G-proteins) (2–4). The biochemical route connecting receptor tyrosine kinases to MAP kinases has been recently identified (1,2). It includes adapter molecules such as Grb2, that help recruit to the membrane a guanine-nucleotide exchange factor, Sos, thereby inducing the exchange of GDP for GTP on Ras, and the consequent activation of a kinase cascade that includes, sequentially, Raf-MEK, and MAP kinase (1,2). The mechanism whereby G protein-coupled receptors activate MAP kinase is still less clear (4).

G proteins are broadly classified into pertussis toxin-sensitive and -insensitive classes. For example, in COS-7 cells lysophosphatidic acid (LPA) potently stimulates MAP kinase through pertussis toxin sensitive G_i-coupled receptors (5). Activation of this and other G_i-linked receptors, such as m2 muscarinic receptors, is believed to stimulate MAP kinase through G_i-$\beta\gamma$ subunits acting on a Ras and Raf-dependent pathway (6,7). Furthermore, recent reports have suggested that $\beta\gamma$ subunits enhance MAP kinase activity by activating Src-like tyrosine kinases, thus leading to the phosphorylation of Shc and the recruitment of Grb2 and Sos complexes to the plasma membrane (7–9). Conversely, activation of MAP kinase through pertussis toxin-insensitive G_q-coupled receptors has been reported to involve a protein kinase C (PKC)-dependent and Ras-independent pathway (10), suggesting a minimal role for $\beta\gamma$ subunits (7). However, we have previously observed that constitutive active forms of $G\alpha_q$ subunit do not activate MAP kinase effectively (6). Furthermore, PKC-down regulation or PKC inhibitors have only a limited effect on MAP kinase activation by G_q-coupled receptors, such as m1 muscarinic receptors (mAChR) or bombesin receptors, in COS-7 and Rat-1 cells, respectively (6,11). Moreover, Rap-1a and dominant negative mutants of Ras abolish m1 and m2-mediated MAPK activation (6). This observation suggests the existence of common signaling pathway in which G_q and G_i coupled receptors activate MAP kinase acting on a Ras-dependent mechanism. Thus, we hypothesized

[1] To whom correspondence should be addressed at Oral and Pharyngeal Cancer Branch, National Institute of Dental Research, National Institutes of Health, 30 Convent Drive, Building 30, Room 212, Bethesda, MD 20892-4330. Fax: (301) 402-0823. E-mail: gutkind@nih.gov.

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that G_q mediated MAP kinase activation might also involve tyrosine kinases of the Src family. To address this issue, we have used the transient expression in COS-7 cells of m1 and m2 mAChRs along with dominant interfering molecules for Src or Src-specific protein kinase inhibitors as a model system to elucidate the biochemical route connecting G protein-coupled receptor to an epitope tagged MAP kinase.

MATERIALS AND METHODS

Reagents. Human recombinant epidermal growth factor (EGF), 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) were obtained from Upstate Biotechnology Incorporated (UBI), and Calbiochem-Novabiochem Corp., respectively. Carbachol and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma Chemical Co.

Cell lines and transfection. COS-7 cells were cultured as previously reported (6). Briefly, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum. Cells were transfected by the DEAE-dextran technique, adjusting the total amount of DNA to 5-10 μg/plate with vector DNA.

Expression Plasmids: Expression plasmids for an epitope tagged MAP kinase (pcDNA3 HA-MAPK), for m1 and m2 mAChRs, β1 and γ2 subunits of heterotrimeric G protein have been described (6). cDNAs for wild type Src (Src WT), kinase inactive Src (Src K295M) (indicated as Src KM) and a double mutant Src exhibiting an open conformation (Src K295M Y527F)(indicated as Src KM YF) (12) were subcloned into expression vector pSM. Expression vectors (pCMV) for Pyk2 and kinase-negative Pyk2 (Pyk K475R) were described (13,14).

MAP kinase assay and Western blot. MAP kinase activity in cells transfected with an epitope-tagged MAPK was determined as described previously, using myelin basic protein (MBP) (Sigma) as a substrate (15). Lysates containing 40 μg of total cellular protein or anti-HA (BABCO) immunoprecipitates were analyzed by Western blotting using anti-HA, anti-Src (UBI) or anti-Pyk2 (Transduction Laboratories) as primary antibodies after SDS-polyacrylamide gel electrophoresis. Immunocomplexes were visualized by enhanced chemoluminescence detection (Amersham Corp.) using anti-mouse or anti-Rabbit IgG coupled to horseradish peroxidase as secondary antibody.

RESULTS

As an approach to study the role of Src-like kinases in signaling from G protein-coupled receptors to MAP kinase, we first explored the ability of a Src-family tyrosine kinase-specific inhibitor, PP1 (16), to affect m1 and m2 induced MAP kinase activation. As shown in Figure 1, PP1 blocked the MAPK response to both the G_q-coupled m1 and the G_i-coupled m2 mAChRs, in a concentration dependent manner. In contrast, PP1 did not affect EGF-induced MAP kinase activation, up to a concentration of 10 μM. These results suggest the involvement of a Src-like kinase in the signaling pathway from G protein-coupled receptor to MAP kinase.

Lately, a novel focal adhesion kinase (FAK) family member, Pyk2 (14,17), and Src kinase families have been reported to play a role in MAP kinase activation by G_i-coupled receptors (8). Thus, we next utilized kinase inactive mutants of Src and Pyk2 to investigate

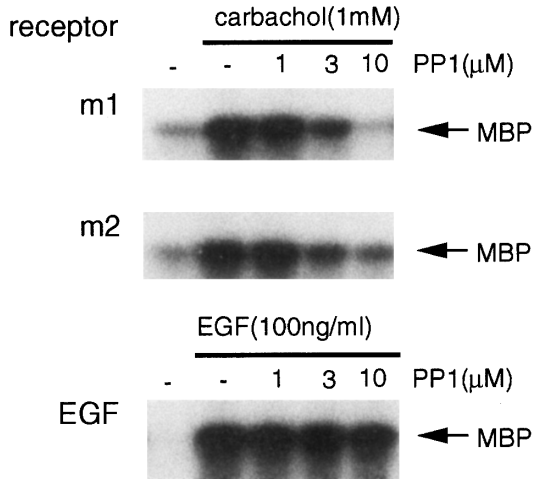

FIG. 1. Inhibition of m1 and m2 mAChR-mediated MAPK activation by PP1. COS-7 cells were transfected with expression plasmids for m1 or m2 mAChRs (3 μg/plate) together with a plasmid expressing an epitope-tagged MAPK (pCDNA3-HA-MAPK, 1μg/plate), or with pCDNA3-HA-MAPK alone. Cultures were treated with the indicated concentration of PP1 for 30 min, followed by stimulation with the addition of carbachol (1mM) or EGF (100 ng/ml) for 5 min, as indicated. MAP kinase activity was determined in the anti-HA immunoprecipitates. Autoradiograms are from a representative experiment, which was repeated 3-5 times with similar results. Position of phosphorylated MBP is indicated.

their relative contribution to m1 and m2 mediated MAP kinase stimulation (Figure 2). In COS-7 cells, neither endogenous Src nor Pyk2 could be detected by Western blot analyses using total cell lysates (Figure 2), but they can be readily detectable in cells transfected with expression plasmids for wild-type and mutant forms of these tyrosine kinases (Figure 2). As shown in the lower panels of figure 2, the wild type forms of Src and Pyk2 did not have any effect on m1 and m2 mediated MAP kinase activation. Pyk2 KR had a marginal suppressing effect on m1 induced MAP kinase activation, without affecting the m2 mediated response. In contrast, both kinase deficient mutants of Src, Src KM and its corresponding open-conformation form, Src KM YF (see below), efficiently diminished MAP kinase activation by m1 and m2 mAChR, although inhibition by Src KM YF was stronger.

The dose dependency of the suppressive effect of the dominant-negative Src was further characterized (Figure 3). Different amounts of Src KM YF expression vector were transfected into COS-7 cells together with HA-MAP kinase and m1 or m2 mAChR, followed by carbachol stimulation for 5 min. The expression of Src increased in a dose-dependent fashion, and HA MAP kinase was comparably expressed. As described above, Src KM YF suppressed the MAP kinase response elicited by both m1 and m2 mAChR, and this inhibitory effect correlated strongly with the expression level of the mutant Src.

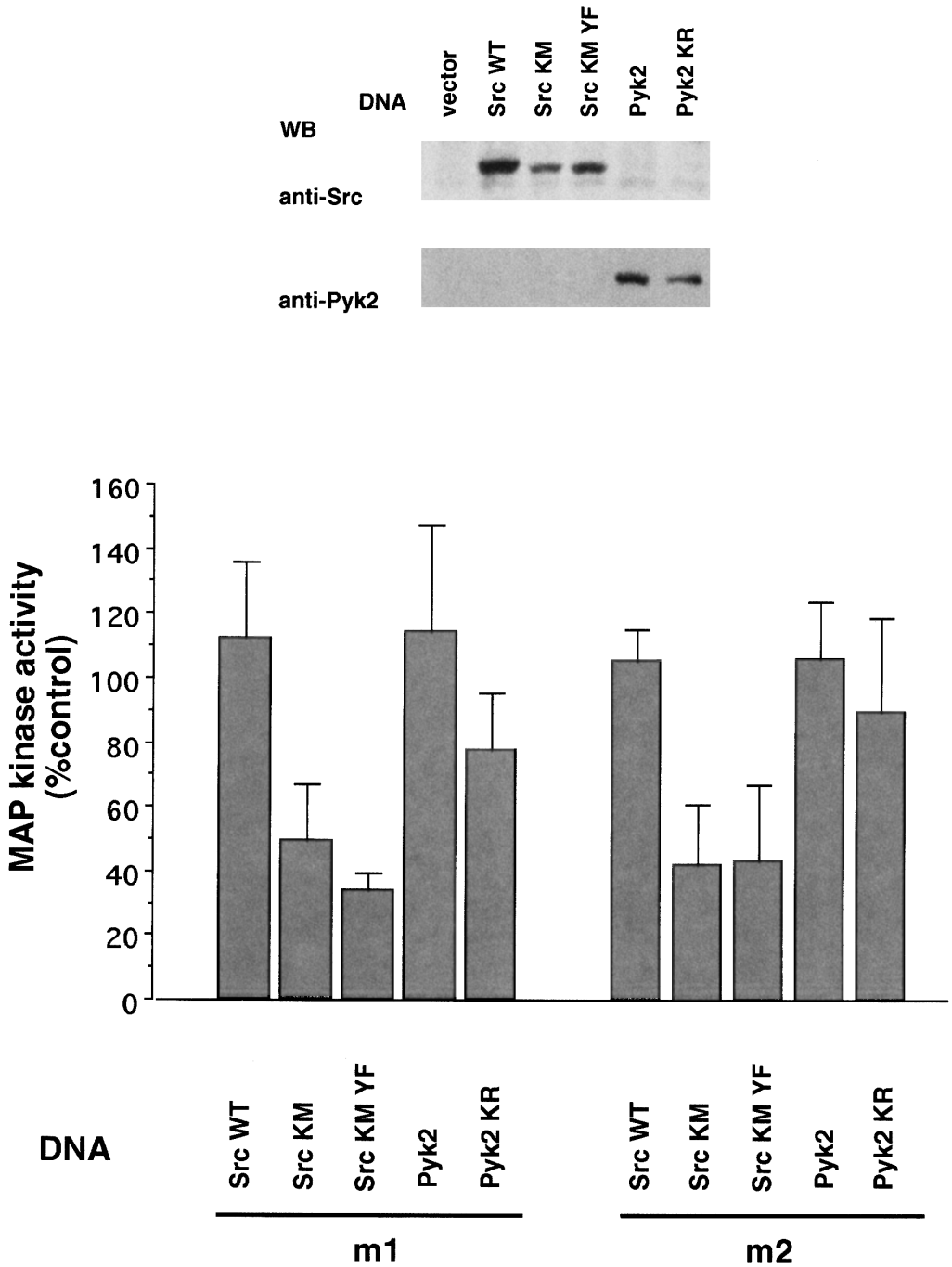

FIG. 2. Effect of kinase inactive Src and Pyk2 on MAP kinase stimulation by m1 and m2 mAChRs. COS-7 cells were transfected with plasmids for m1 or m2 mAChRs (3μg/plate) together with a plasmid expressing pCDNA3-HA-MAPK (1μg/plate) and plasmids for the indicated kinases (1 or 2 μg/plate), followed by stimulation with addition of carbachol (1mM) for 5 min. The upper panel is showing the expression of the indicated kinases. The lower panel is showing the relative MAP kinase activity compared to that of controls. Data represent the mean ± S.E. of four to five independent experiments.

Next, we tested the effects of the Src KM YF mutant on the time course of the MAP kinase response to m1 and m2 mAChR stimulation (Figure 4). In m1 mAChR stimulated cells, MAP kinase was activated in a sustained manner, but in m2 mAChR expressing cells, MAP kinase stimulation showed a transient pattern, returning to nearly the basal level after 30 min. In both cases, Src KM YF inhibited MAPK activation without changing the original pattern, together suggesting that in spite of the diversity of signaling pathways triggered

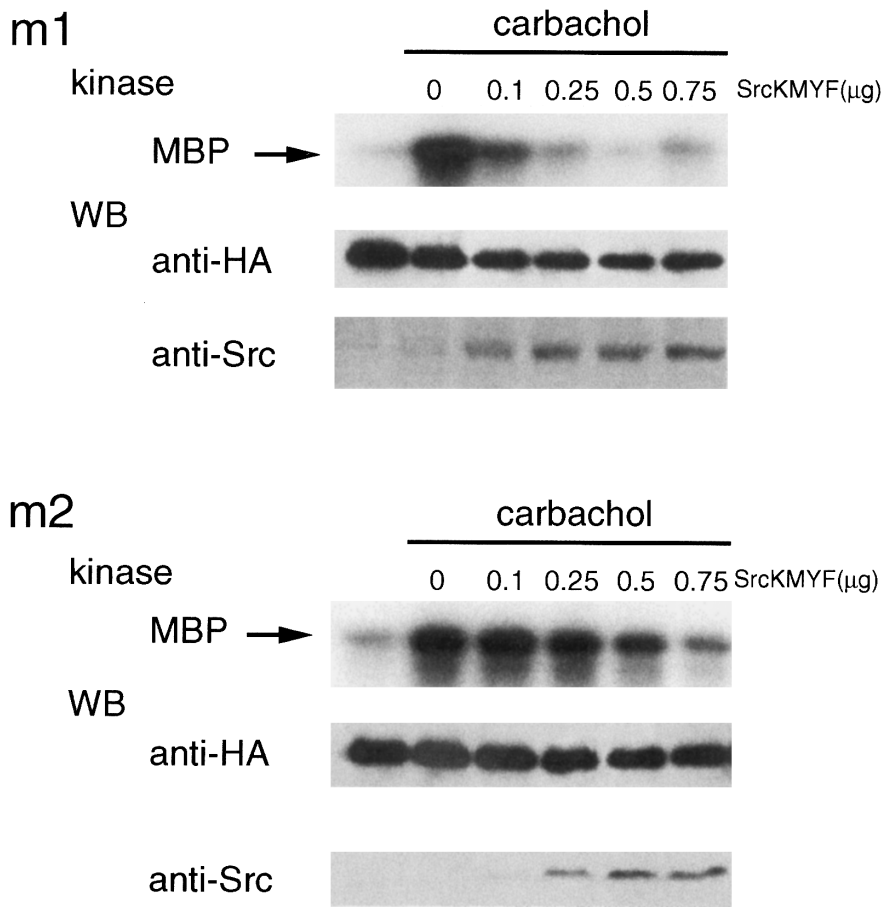

FIG. 3. The expression of dominant-negative Src diminishes m1 and m2 mAChR mediated MAP kinase activation. COS-7 cells were transfected with plasmids for m1 or m2 mAChRs (3 μg/plate) together with a plasmid expressing pCDNA3-HA-MAPK (1μg/plate) and the indicated amount of pSM Src KM YF, followed by stimulation with addition of carbachol (1mM) for 5 min. Kinase reactions and Western blot analyses with anti-HA were performed in anti-HA immunoprecipitates from the same lysates. Western blot analyses for Src were performed using 40 μg of total cellular protein from the same lysates. This experiment was repeated 3 times with nearly identical results.

by the m1 and the m2 class of mAChRs, both converge at the level of Src to stimulate MAP kinase.

In this regard, whereas m1 receptors are typical of those coupled to phospholipase C activation through G proteins of the G_q family, m2 is known to couple through G_i to a number of effector pathways, including the inhibition of adenylyl cyclase (18). In COS-7 cells, MAP kinase activation *via* G_i coupled receptors is mediated largely by $\beta\gamma$ subunits. On the other hand, G_q coupled receptors were reported to activate MAP kinase through PKC, independent of Ras (7,10). Thus, to explore the common mechanism by which m1 and m2 activate MAP kinase, we studied the effect of Src KM YF expression on MAP kinase stimulation by overexpression of $\beta\gamma$ subunits and by treatment with TPA (Figure 5). The overexpression of $\beta\gamma$ subunits effectively activated MAP kinase, and cotransfection of Src KM YF suppressed this activation by more than 60%. In contrast, Src KM YF had a minimal effect on TPA-induced MAP kinase activation. Taken together, we concluded that Src KM YF inhibits $\beta\gamma$ subunits-dependent component of both m1 and m2-induced MAP kinase activation, without affecting the PKC-dependent pathway.

DISCUSSION

We have utilized m1 and m2 mAChR as a model system for the study of proliferative signaling through G_q and G_i-coupled receptors, and reported that both of them strongly activate MAP kinase (6,19). In previous study, we have provided evidence that $\beta\gamma$ subunits released from both G_i and G_q are critical initiators of this signaling pathway (6). Several tyrosine kinases including the Src family, Pyk2 and Syk have been proposed to be mediators of Ras activation from G_i-coupled receptors (7,8,20–22). In the present study, we have shown that the G_q-coupled, m1 receptor-initiated MAP kinase stimulation is also Src-kinase dependent in COS-7 cells, and that Src-like kinases act probably

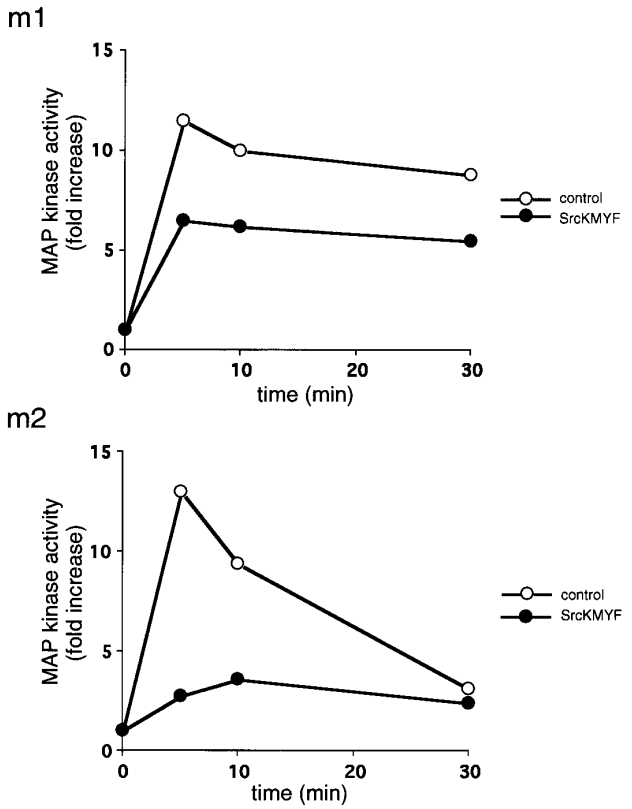

FIG. 4. Effect of the dominant-negative Src on the time course of m1 and m2 mAChR mediated MAP kinase activation. COS-7 cells were transfected with plasmids for m1 or m2 mAChRs (3μg/plate) together with a plasmid expressing pCDNA3-HA-MAPK(1μg/plate) and pSM Src KM YF (1μg/plate) and stimulated for indicated time with carbachol (1mM). Data are expressed as fold increase in MAP kinase activity with respect to non-stimulated cells.

downstream from $\beta\gamma$ subunits released from both G_q and G_i.

First, we tested the effect of PP1, which is a selective Src-family kinase inhibitor (16), on m1, m2 and EGF induced MAP kinase activation. This compound is 40-50 fold more potent suppressing Lck and Fyn kinase activity than the EGF tyrosine kinase receptor *in vitro*, and suppresses CD3 mediated-lymphocyte proliferation at lower μM concentration (IC50 $\sim$0.5 μM), whereas it affects phorbol 12-myristate 13 acetate (TPA) and IL-2 stimulated proliferation at a much higher concentration (IC50 $\sim$26 μM) (16). These observations suggest that the concentrations of PP1 used in this study (up to 10 μM) are effectively diminishing the activity of Src-like kinases without inhibiting other kinases, such as the EGF receptor (Figure 1) and PKC (not shown). Thus, the result that PP1 strongly suppressed both m1 and m2, but not EGF-induced MAP kinase activation, supports the hypothesis that Src-family members are involved in both m1 and m2 mAChR initiated signaling to MAP kinase.

Recently, a new member of the FAK family of non-receptor tyrosine kinases, Pyk2, has been cloned and proposed to mediate G_i and G_q initiated MAP kinase activation in cooperation with Src in PC12 cells (17). However, in COS-7 cells a similar kinase inactive Pyk2 did not exhibit an inhibitory effect on m2 mediated MAP kinase activation, and had only a very limited effect in m1 induced-MAP kinase stimulation. Furthermore, it has been proposed that in HEK-293 cells the calcium-dependent activation of Pyk2 or Pyk2-like kinase plays an important role in Src activation (23). However, in COS-7 cells elevation of intracellular calcium with ionomycin does not result in MAP kinase stimulation (24,25; and data not shown), nor we could observe MAP kinase activation by constitutive active α_q (6). Thus, taken together Pyk2 does not appear to play a critical role in MAP kinase stimulation by G proteins in COS-7 cells.

In contrast, both Src KM and Src KM YF mutants effectively diminished m1 and m2 initiated MAP kinase activation in COS-7 cells. These observations promoted us to speculate that Src-like kinases are involved in a common mechanism by which m1 and m2 mAChR activate MAPK in these cells. Based upon the finding that Src KM YF diminishes $\beta\gamma$ but not TPA-induced MAP kinase stimulation, we can conclude that the common mechanism is likely to involve $\beta\gamma$ complexes rather than PKC or other signaling molecules downstream from $G\alpha_q$ or $G\alpha_i$. Interestingly, because Src KM YF mutant takes an open conformation due to the replacement of a carboxyl-terminal tyrosine for phenylalanine (26), it is expected to be a more potent dominant-

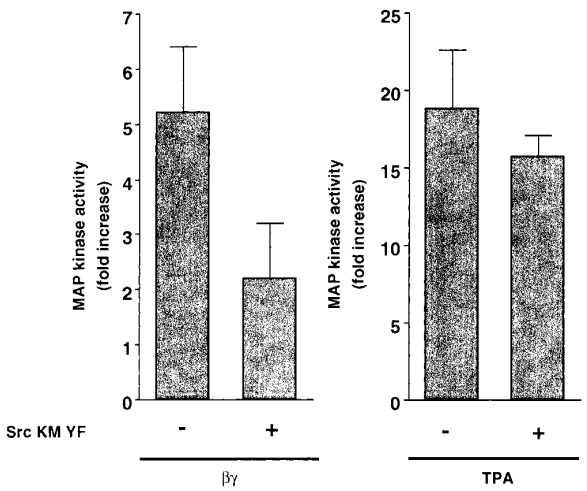

FIG. 5. Effect of dominant-negative Src on $\beta\gamma$ subunits and TPA-induced MAP kinase activation. COS-7 cells were transfected with pCDNA3-HA-MAPK (1μg/plate) alone for TPA stimulation, or together with plasmids for β1 and γ2 (2μg/plate each), with or without pSM Src KM YF (0.75 μg/plate). Cells were stimulated with TPA (100 ng/ml) for 5 min (right panel) or untreated for $\beta\gamma$ overexpressing cells (left panel). Data represent the mean $\pm$ S.E. of three independent experiments, expressed as fold increase with respect to untransfected or non-stimulated cells.

negative inhibitory molecule. As such, Src KM YF inhibited the MAP kinase response to m1 stimulation more strongly than Src KM. That might help explain why others, using the Src KM mutant, have not been able to observed the blockade of MAP kinase activation by LPA in COS-7 and Rat-1 (27).

On the other hand, although we have demonstrated that the dominant-negative Src effectively suppresses MAP kinase activity induced by m1 and m2 mAChRs, it does not necessarily indicate that Src itself is involved in the signaling pathway from G_q and G_i to MAP kinase in COS-7 cells. In this regard, we have observed that COS-7 cells express high levels of Fyn and Lyn, which are closely related to Src. As there is a functional redundancy among Src kinase family members (28), and Src itself is poorly expressed in COS-7 cells, our observations strongly suggest that these or other Src-like tyrosine kinases mediate MAP kinase activation by G protein-coupled receptors in COS-7 cells.

In summing up, there may be a complex kinase network downstream of G proteins, and the kinases mediating MAP kinase activation by G protein-coupled receptors may be distinct when comparing different cell types. Our data suggest that, in COS-7 cells, Src like kinases play an important role downstream of $\beta\gamma$ subunits released, not only from activated GI, but also from G_q to relay the signal from G protein-coupled receptors to the MAP kinase pathway.

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