Gq-Mediated Activation of c-Jun N-Terminal Kinase by the Gastrin-Releasing Peptide-Preferring Bombesin Receptor Is Inhibited upon Costimulation of the Gs-Coupled Dopamine D1 Receptor in COS-7 Cells

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ABSTRACT

G protein-coupled receptors (GPCRs) of Gs- or G q-coupling specificity are effectively linked to activation of the c-Jun N-terminal kinase (JNK) cascade. However, little is known with regard to the regulation of JNK by G s-coupled receptors. In this report, we used COS-7 cells transfected with the dopamine D1 receptor (D1R) to illustrate the signaling mechanism for Gs-mediated JNK activation. Stimulation of D1R triggered a weak but significant elevation of JNK activity in a time- and dose-dependent manner. This D1R-mediated JNK activation required the participation of Gβγ, Src-like kinases, and small GTPases, whereas disruptions of cAMP-, phosphoinositide-3-kinase-, and epidermal growth factor receptor-mediated signaling had no effect. Costimulation of D1R with GPCRs of other coupling specificities resulted in differential activation profiles of JNK. Activation of Gs-coupled D1R weakly potentiated the JNK activation induced by the Gs-coupled opioid receptor-like receptor, but it exhibited a significant inhibitory effect on the kinase activity triggered by the Gq-coupled gastrin-releasing peptide-preferring bombesin receptor (GRPR). Administration of Sp-adenosine-3',5'-cyclic monophosphorothioate triethylamine (a cAMP analog that mimics the Gs/cAMP signal) also suppressed the JNK activation mediated by Gs-coupled GRPR, as well as the Ca2+-induced kinase activation upon thapsigargin treatment. Moreover, the Ca2+ signal from GRPR synergistically potentiated the D1R-triggered cAMP elevation when the two receptors were stimulated simultaneously. Taken together, our results demonstrated that stimulation of Gs-coupled receptors in COS-7 cells not only enhanced the JNK activity, but also exhibited a “tuning” effect on the kinase activation mediated by GPCRs of other coupling specificities.

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ABBREVIATIONS: MAPK, mitogen-activated protein kinases; GRPR, gastrin-releasing peptide-preferring bombesin receptor; JNK, c-Jun N-terminal kinase (JNK), and p38 (Widmann et al., 1999). Among them, the biological functions of JNK are relatively diverse, ranging from cell proliferation, to differentiation, to survival, to apoptosis (Dunn et al., 2002). It is believed that JNK may exhibit its multifunctional characteristics by phosphorylating the transcription factors (e.g., c-Jun and ATF-2) and hence modulating cellular gene expression (Dunn et al., 2002).

G protein-coupled receptors (GPCRs) are a major group of transmembrane receptors for detecting extracellular signals (Pfleger and Eidne, 2005). GPCRs, particularly those selectively coupled to the Gs and Gq families of G proteins, are...
efficiently linked to the activation of JNK. G<sub>I</sub>-coupled receptors mainly require a Gβγ/Src-dependent mechanism to stimulate the JNK cascade (Chan and Wong, 2004a; Kam et al., 2004), whereas G<sub>S</sub>-coupled receptors use both Gβγ/Src and Ca<sup>2+</sup> signals to regulate the kinase activity (Chan and Wong, 2004b). In addition to the participation of Src-like kinases, functional activities of PI3K isoforms and the transactivation of epidermal growth factor receptors (EGFRs) have been proposed as alternative routes for the Gβγ-mediated pathway (Lopez-Ilasaca et al., 1998; Murga et al., 2000; Pierce et al., 2001). Despite the possible differential involvements of signaling intermediates, Gβγ seems to play an important role for both G<sub>S</sub> and G<sub>I</sub>-mediated JNK activation in response to stimulation of GPCRs.

Receptors coupled to the G<sub>S</sub> family of G proteins are characterized by their abilities to trigger adenylyl cyclase-mediated cAMP formation (Bal forskoth et al., 1986). Among all GPCRs that show coupling preferences toward a particular G protein family, much less is known with regard to the stimulation of JNK by G<sub>I</sub>-coupled receptor. It has been demonstrated that activation of dopamine D<sub>1</sub> receptors in 5K-N-NC human neuroblastoma cells is linked to increased JNK activity in a cAMP and PKA-dependent manner (Zhen et al., 1998) and is therefore readily suppressed by pretreatment with Rp-cAMPs or H89, which specifically inhibit the cAMP/PKA signaling. Another report on the β<sub>2</sub>-adrenergic receptor in DDT1 MF-2 smooth muscle cells also supports this idea and further suggests the possible involvement of Rho family GTPases in the G<sub>S</sub>-mediated JNK stimulation (Yamauchi et al., 2001). In contrast, numerous studies have demonstrated that G<sub>S</sub>-coupled receptors generally lack the ability to stimulate JNK. For instance, activation of G<sub>S</sub>-coupled adenosine A2A receptor in HMC-1 human mast cells does not enhance the JNK activity (Feoktistov et al., 1999). Likewise, studies of Chinese hamster ovary cells overexpressing G<sub>S</sub>-coupled corticotrophin-releasing factor receptors (Rossant et al., 1999) or β-adrenergic receptors (Gerhardt et al., 1999) illustrate that these receptors are incapable of stimulating JNK activity upon specific agonist treatment.

Despite the inability of many G<sub>S</sub>-coupled receptors to stimulate JNK, cAMP has been suggested as an activator for JNK activity. Administration of cAMP analogs (e.g., 8-bromo-cAMP and 3-O-methyladenosine-3′,5′-cyclic monophosphorothioate) and adenylyl cyclase stimulants (e.g., forskolin) has been reported to activate the JNK cascade in DDT1 MF-2 smooth muscle cells (Yamauchi et al., 2001) and MC3T3-E1 preosteoblast cells (Kanno et al., 2004), respectively. However, other groups showed that these cAMP-elevating agents have no effect on JNK activation in hepatocytes (Reinehr et al., 2004) and are even associated with an inhibitory effect on the JNK activity triggered by epidermal growth factor (McCawley et al., 2000). The inhibitory effect of cAMP on the growth factor-induced JNK activation implies that activation of G<sub>q</sub>-coupled receptors, or administration of cAMP-elevating agents, may suppress the kinase activation triggered by GPCRs of other coupling specificities.

We have demonstrated previously that transfected COS-7 cells transiently expressing GPCRs are useful cellular models to study the activation of JNK mediated by G<sub>S</sub> and G<sub>q</sub>-coupled receptors (Chan and Wong, 2000, 2004a; Chan et al., 2002), with the experimental results highly consistent with those obtained from endogenous cellular systems (Kam et al., 2003, 2004; Chan and Wong, 2004b). In this report, we used COS-7 cells transfected with G<sub>S</sub>-coupled dopamine D<sub>1</sub> receptor (D<sub>R</sub>) to illustrate the signaling mechanism for JNK activation and further investigated the effects of costimulation of G<sub>S</sub>-coupled receptor with G<sub>I</sub>- or G<sub>q</sub>-coupled receptors on the JNK activity. Our results suggest that stimulation of G<sub>S</sub>-coupled receptor in COS-7 cells not only enhances the JNK activity, but also exhibits differential regulatory effects on the kinase activation mediated by G<sub>S</sub>- and G<sub>q</sub>-coupled receptors.

Materials and Methods

Materials. The cDNAs encoding the GRPR and ORL-R were kindly provided by Dr. J. Battey (National Institutes of Health, Bethesda, MD) and Dr. Gang Pei (Shanghai Institutes for Biological Sciences, Shanghai, Hong Kong), respectively. The plasmid encoding D<sub>R</sub> was obtained from Guthrie Research Institute (Sayre, PA). The cDNAs of dominant-negative mutants RasS17N and RacT17N were generous gifts from Dr. Eric Stanbridge (University of California, Irvine, Irvine, CA), RhoT19N and Cdc42T17N were provided by Dr. Marc Symons (Picoiver Institute for Medical Research, New York, NY). The plasmid of HA-tagged JNK was donated by Dr. Tatyana Voyno-Yasenetskaya (University of Illinois, Chicago, IL). [γ<sup>32</sup>P]ATP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Pertussis toxin and 12CA5 (Anti-HA) antibody were purchased from List Biological Laboratories Inc. (Campbell, CA) and Roche Molecular Biochemicals (Indianapolis, IN), respectively. Phospho-CREB antibody and CREB antibody were obtained from Cell Signaling Technology (Beverly, MA). Cell culture reagents including Lipofectamine PLUS were obtained from Invitrogen (Carlsbad, CA). Bomeisin, dopamine, and nociceptin were purchased from Sigma-Chemical (St. Louis, MO). Thapsiargin, BAPTA-AM, AG1478, radiocilic, wortmannin, calphostin C, Rp-cAMPs, and Sp-cAMPs were obtained from Calbiochem (San Diego, CA).

Cell Culture and Transfection. Green monkey kidney fibroblast COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 50 units/ml penicillin, and 50 μg/ml streptomycin and were maintained at 37°C in an environment of 5% CO<sub>2</sub>. COS-7 cells were transfected to six-well plates at 4 × 10<sup>5</sup> cells/well (for JNK assay) or to 12-well plates at 1.5 × 10<sup>6</sup> cells/well [for adenylyl cyclase and phospholipase C assays. Transfection was performed by means of Lipofectamine PLUS reagents following the supplier's instructions.

In Vitro JNK Assay. Thirty-six hours after transfection, COS-7 cells were serum-starved overnight and then treated with various inhibitors if necessary. The cells were then stimulated with specific agonists for the indicated durations, and the assays were terminated by washing the cells with phosphate-buffered saline, followed by the addition of 500 μl of ice-cold detergent-containing lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM Na<sub>P</sub>O<sub>4</sub>, 1% Triton X-100, 1 mM dithiothreitol, 200 μM Na<sub>3</sub>VO<sub>4</sub>, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml apro tin, and 0.7 μg/ml pepstatin). Lysates obtained were subjected to JNK assay as described previously (Chan and Wong, 2000). Fifty micro liters of each supernatant was used for the detection of JNK-α or JNK-β expression, and the remaining (450 μl) was incubated for 1 h at 4°C with 12CA5 (Anti-HA) antibody (2 μg/sample), followed by incubation with 30 μl of protein A-agarose (50% slurry) at 4°C for 1 h. The resulting immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer [40 mM HEPES, pH 8.0, 5 mM Mg<sub>(CH<sub>3</sub>)<sub>2</sub>O<sub>4</sub>], 1 mM EGTA, 1 mM dithiothreitol, and 200 μM Na<sub>3</sub>VO<sub>4</sub>]. Washed immunoprecipitates were resuspended in 40 μl of kinase assay buffer containing 5 μg of GST-c-Jun per reaction, and the kinase reactions were initiated by the addition of 10 μl of ATP buffer (50 μM ATP containing 2 μCi of [γ<sup>32</sup>P]ATP per sample). After 30-min incubation at 30°C with occasional shaking, the reactions
were terminated by 10 μl of 6× sample buffer, and the samples were resolved by 12% SDS-PAGE. The radioactivity incorporated to GST-c-Jun was detected by autoradiogram, and the signal intensity was quantified by PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK; 445 SI).

PLC Assay. One day after transfection, COS-7 cells were labeled for 18 h with 0.75 ml of inositol-free DMEM containing [myo-3H]inositol (5 μCi/ml) and 10% FCS (v/v) followed by serum starvation for 18 h. The cells were then pretreated in assay medium (20 mM HEPES-buffered DMEM with 20 mM LiCl) for 10 min and subsequently stimulated in the presence or absence of indicated drugs for 30 min at 37°C. The reactions were terminated by the aspiration of drug-containing medium, followed by the addition of ice-cold 20 mM formic acid solution. After 1-h incubation at 4°C, cell extracts were subjected to ion-exchange chromatography as described previously (Chan and Wong, 2004a).

Adenylyl Cyclase Assay. Transfected COS-7 cells were labeled with 2 μCi/ml [3H]adenine in DMEM (10% FCS, v/v) for 18 h. After serum starvation for 18 h, cells were treated with the assay medium (DMEM containing 20 mM HEPES and 1 mM 1-methyl-3-isobutylxanthine) in the presence or absence of indicated drugs for 30 min at 37°C. The reactions were terminated by aspiration of drug-containing medium, followed by the addition of ice-cold 5% (v/v) trichloroacetic acid solution with 1 mM ATP (1 ml/well) and kept at 4°C for 1 h. Intracellular levels of [3H]cAMP were determined by sequential chromatography, as described previously (Chan and Wong, 2004a).

**Results**

**Time/Dose-Dependencies of D1R-Mediated JNK Activation.** We have demonstrated previously that COS-7 cells transiently expressing D1R serve as reliable models for studying Gs-mediated signaling, which is linked to a weak but significant activation of JNK upon dopamine treatment (Chan and Wong, 2004a). Hence, we began our study by challenging D1R transfectants with a fixed dose of dopamine for increasing durations, or with increasing doses for a fixed period. The D1R-induced JNK activation gradually increased within the first 15 min of dopamine treatment, reaching the maximal level at 30 min, and decreased to a near basal level after 60 min of receptor stimulation (Fig. 1A). Increasing agonist concentrations also gradually enhanced the kinase activity, with the maximal effect occurring at 10 μM dopamine (Fig. 1B). To examine whether this induced kinase activation was contributed by the Gs/cAMP signaling, the cells were pretreated with Rp-cAMPs, a cell-permeable cAMP...
analog having inhibitory effects on cAMP/PKA-regulated cellular events. Our results showed that Rp-cAMPs (100 μM) had no significant inhibitory effect on the D1R-induced JNK activity (Fig. 1C). Further investigation on the Gs-coupled lutropin hormone receptor (LHR) transiently expressed in COS-7 cells also produced similar results (Fig. 1D). Our control experiment indicated that Rp-cAMPs (100 μM) effectively suppressed the cAMP/PKA-mediated phosphorylation of CREB upon stimulation with dopamine (Fig. 1E).

**Signaling Intermediates of D1R-Mediated JNK Activation.** Activation of JNK in response to Gβγ and Gαs-coupled receptors seems to depend on Gβγ subunits (Chan and Wong, 2004a,b). Such a response can be suppressed by transducin, which acts as an effective scavenger to remove free Gβγ subunits released upon G protein activation. For COS-7 cells expressing Gαs-coupled D1R, the dopamine-induced JNK activation was almost completely inhibited when transducin was coexpressed in the cells (Fig. 2A). This is consistent with a previous report showing that Gβγ subunits are better activators than the α-subunit of Gαs (Gαs) in terms of JNK activation (Coso et al., 1996). To reveal the identities of Gβγ-regulated intermediates for the D1R-mediated JNK activation, target-specific inhibitors (radicicol and PP1 for Src-like kinases, wortmannin for PI3K isoforms, and AG1478 for EGFR) were used. Pretreatment of radicicol (Fig. 2A) and PP1 (data not shown) significantly suppressed the D1R-mediated JNK activation, whereas wortmannin and AG1478 had no effect on the induced kinase response (Fig. 2A). Intermediates downstream of Src-like kinases include guanine nucleotide exchange factor (GEF)-regulated GTPase activities of the Ras and Rho family members (Kiyono et al., 2000), and the study of their involvements is usually performed by expression of the corresponding dominant-negative mutants of these small GTPases (i.e., RasS17N, RacT17N, RhoT19N, and Cdc42T17N). When D1R was coexpressed with either one of these mutants followed by subsequent dopamine treatment, the induced JNK activation was significantly inhibited in the presence of RasS17N, RacT17N, or Cdc42T17N, but not RhoT19N (Fig. 2B). Activation of JNK by Gαs-coupled LHR

![Fig. 2](https://example.com/figure2.png)

**Fig. 2.** Gβγ subunits, Src-like kinases, and small GTPases serve as important intermediates for D1R-mediated JNK activation. COS-7 cells were transfected with the cDNAs of JNK-HA and the Gαs-coupled D1R (A and B). The cells were either coexpressing transducin (Gαs) for Gβγ scavenging; pretreated with radicicol (10 μM, 1 h for Src-like kinases), wortmannin (100 nM, 15 min for PI3K), or AG1478 (500 nM, 30 min for EGFR) for specific signal disruption (A); or transfected with different dominant-negative mutants of small GTPases (B) before agonist treatment. JNK assay was performed as described under Materials and Methods. Values shown represent the mean ± S.E. from three to six separate experiments. *, administration of dopamine induced significant activation of JNK compared with the corresponding basal. #, removal of Gβγ by transducin, pretreatment with radicicol, and the presence of RasS17N, RacT17N, and Cdc42T17N significantly inhibited the JNK activation compared with the control group (Bonferroni paired t test, P < 0.05).
was also characterized by dependencies on GBγ, Src-like kinases, and small GTPases (data not shown).

**Differential Regulatory Effects of G_s-Coupled D_1R on the JNK Activation Triggered by G_s-Coupled ORL_1R and G_q-Coupled GRPR.** The preceding experiments demonstrated that G_s-coupled D_1R activated JNK in a GBγ-, Src-like kinase-, and small GTPase-dependent manner (Fig. 2). Such dependencies have also been observed in our previous studies for the same kinase activation mediated by G_s-coupled ORL_1R (Chan and Wong, 2000) and G_q-coupled GRPR (Chan and Wong, 2004b). To investigate the integrated JNK activities upon GPCR coactivation, we cotransfected G_s-coupled D_1R with either G_s-coupled ORL_1R (Fig. 3A) or G_q-coupled GRPR (Fig. 3B) in COS-7 cells, followed by individual or coadministration of corresponding agonists. For cells coexpressing D_1R and ORL_1R, individual treatment with appropriate agonists (dopamine for D_1R and nociceptin for ORL_1R) triggered JNK activation to ~1.5- and ~2.0-fold, respectively (Fig. 3A), compared with the corresponding basal (defined as 1.0-fold). These magnitudes of JNK activities were consistent with the results shown in Fig. 1 for D_1R, and with our previous report for ORL_1R (Chan and Wong, 2000) using COS-7 cells transiently expressing either one of the receptors. Costimulation of both D_1R and ORL_1R resulted in JNK activation in a roughly additive manner (Fig. 3A). These results in conjunction with our previous findings (Chan and Wong, 2000) showed that although D_1R and ORL_1R use a similar mechanistic pathway (GBγ → Src-like kinase → small GTPase → JNK cascade), D_1R remained capable of complementing the kinase response triggered by ORL_1R. To reveal whether G_s-coupled D_1R also exhibits a similar effect on the JNK activity stimulated by G_q-coupled GRPR, COS-7 cells were cotransfected with these two receptors. Activation of D_1R by dopamine retained the ability to activate JNK weakly, and the GRPR agonist (i.e., bombesin) was still capable of triggering a 6- to 7-fold kinase activity (Fig. 3B), similar to our results in the previous report (Chan and Wong, 2004b). It was unexpected that when both D_1R and GRPR were costimulated in the same cells, the induced JNK activation was significantly decreased compared with the GRPR-mediated response (Fig. 3B). These results suggested that even though the G_s-coupled D_1R is linked to activation of JNK activity, it possesses an inhibitory effect on the same kinase activity triggered by G_q-coupled GRPR in COS-7 cells.

**Modulation of D_1R-Mediated Adenylyl Cyclase Activation by G_s-Coupled ORL_1R and G_q-Coupled GRPR.** The differential regulatory effects of D_1R on the ORL_1R-and GRPR-induced JNK activity (Fig. 3) implied that, although G_s-, G_q-, and G_g-coupled receptors use a common GBγ-dependent mechanism to stimulate the JNK activity, G protein subfamily-specific signaling may influence each other and enable an “alternative” regulatory route for their integrated activation of JNK.

Measurements of the G_s/adenylyl cyclase-mediated cAMP elevation may serve as a useful means for examining this possibility. In COS-7 cells coexpressing G_s-coupled D_1R and G_q-coupled ORL_1R, administration of dopamine triggered an elevated cAMP level, whereas nociceptin treatment induced no observable changes in cAMP production (Fig. 4A). When both of these agonists were coadministered, a net increase of cAMP formation was produced, which was associated with a significant inhibition compared with the dopamine-induced activity (Fig. 4A). This result agreed with the classic G protein signaling model that the inhibitory G_i signal opposed the stimulatory G_s signal on the adenylyl cyclase-mediated cAMP formation.

Likewise, when COS-7 cells coexpressing G_s-coupled D_1R and G_q-coupled GRPR were stimulated by their specific agonists, dopamine treatment was capable of triggering enhanced cAMP formation, whereas bombesin did not significantly stimulate this activity (Fig. 4B). However, when dopamine and bombesin were coadministered, the induced cAMP production was nearly doubled compared with the dopamine effect (Fig. 4B). This indicated that the G_i signal...
from GRPR synergizes with the Gα-mediated adenylyl cyclase activation by D1R. To investigate the mechanism that gave rise to this synergistic response, a series of experiments was performed by target-specific inhibition on the Gα signaling or by testing the Gβγ-dependence of adenylyl cyclase activation. For cells cotransfected with transducin, removal of Gβγ subunits (released from Gs and Gq) was incapable of eliminating the synergistic response upon costimulation of D1R and GRPR (Fig. 4C). When the cells were pretreated with calphostin C to inhibit PKC functions, dopamine treatment remained capable of increasing the cAMP level, and the subsequent synergistic effect with bombesin was still present (Fig. 4D). Chelation of intracellular Ca2⁺ by BAPTA-AM did not affect the dopamine or bombesin responses, but it almost completely eliminated the synergistic adenylyl cyclase activation by returning the cAMP level to nearly the same level as the dopamine treatment alone (Fig. 4E). The above results suggested that the Gq-induced Ca2⁺ activity is important for the GRPR-mediated potentiation of the D1R-triggered cAMP elevation. To provide direct evidence for the synergism of Gs-mediated cAMP formation in response to the elevated Ca2⁺ activity, COS-7 cells expressing D1R alone were stimulated with dopamine and thapsigargin (an extensively used agent for elevating intracellular Ca2⁺ level) separately or simultaneously. Again, dopamine treatment significantly enhanced the cAMP level, but thapsigargin failed to do so, whereas their coapplication generated synergistic adenylyl cyclase activation (Fig. 4F). These results showed that the Gα-coupled GRPR may use a Ca2⁺ signal to potentiate the adenylyl cyclase activity induced by Gα-coupled D1R.

Costimulation of Gα-Coupled D1R with Either Gβγ-Coupled ORL1R or Gq-Coupled ORL1R Had No Effect on the PLC Activity. Gβγ-coupled receptors are known to use both Gβγ- and PLC-dependent pathways to regulate the JNK activity (Chan and Wong, 2004b). Thus, we examined whether signal integration might have occurred along the Gα/PLC pathway. In COS-7 cells coexpressing Gα-coupled GRPR and Gq-coupled D1R, treatment with bombesin stimulated IP₃ formation, whereas activation of D1R by dopamine neither stimulated PLC nor potentiated the GRPR-triggered response (Fig. 5A). Because the activities of PLC/B-isozymes may be potentiated by Gβγ subunits released upon Gα activation (Chan et al., 2000), a similar assay was hence performed with COS-7 cells coexpressing Gα-coupled D1R and Gi-coupled ORL1R to determine whether cooperative signaling between Gα and Gi is capable of elevating the IP₃ formation. However, irrespective of whether these cells were treated with dopamine and nociceptin individually or simultaneously, no significant enhancement of IP₃ formation was observed (Fig. 5B).
cAMP Serves as a Suppressor of Gq-Mediated JNK Activation in COS-7 Cells. The preceding experiments demonstrated that costimulation of D1R and GRPR was associated with a synergistic elevation of cAMP (Fig. 4B) but a diminished magnitude of JNK activation (Fig. 3B). To examine whether the cAMP signal can suppress G1-mediated JNK activation, COS-7 cells were transfected with D1R and GRPR and then treated with Sp-cAMPs (a cell-permeable analog with stimulatory effect on cAMP-mediated signaling) in the absence or presence of bombesin. Sp-cAMPs itself did not stimulate JNK; instead, it significantly suppressed the bombesin-mediated JNK activation (Fig. 6A). The inability of Sp-cAMPs to suppress the JNK activation mediated by G1-coupled ORL1R suggested that cAMP signaling specifically inhibited G1- but not G1-mediated JNK activation in COS-7 cells (Fig. 6B). Further investigations showed that this selective inhibitory effect on GRPR-mediated JNK activation showed a dose-dependent character toward Sp-cAMPs (Fig. 7). In addition to the Gβγ-dependent pathway, G1-coupled receptors also require a Ca2+ component to stimulate the JNK cascade (Chan and Wong, 2004b). It is possible that cAMP exerts its inhibitory effect on the Ca2+-mediated pathway, resulting in a G1-mediated suppression of the Gq-induced JNK activation. In fact, our previous report has already demonstrated that when transfected COS-7 cells were treated with thapsigargin to induce a Ca2+-dependent JNK activation, cotreatment with increasing concentration of Sp-cAMPs resulted in a gradual decrease of kinase activities (Chan and Wong, 2004a). All of these data support the idea that cooperation of Gs and Gq signaling may result in decreased Gq-mediated JNK activation, which is primarily caused by a suppressive effect of cAMP on the Ca2+ signaling component of the Gq-mediated JNK activity.

Disruption of Ca2+ Signaling by BAPTA-AM Suppressed the D1R/GRPR-Mediated JNK Activity to the D1R/ORL1R-Induced Level. As discussed earlier, Gs-, Gi-, and Gq-coupled receptors use the Gβγ-dependent pathway to regulate the activity of JNK cascade in COS-7 cells, with the Gq-mediated response associated with an additional involvement of Ca2+ signaling. These characteristics imply that in the absence of the elevated Ca2+ signal upon Gq activation, costimulation of Gq and Gs should be similar to that of Gi and Gs, in terms of JNK activation mediated by Gβγ subunits from two different G protein subfamilies. To examine this

![Fig. 5.](image)

Costimulation of G1-coupled D1R with either G1-coupled GRPR or G1-coupled ORL1R had no effect on PLC. COS-7 cells coexpressing D1R with either GRPR (A) or ORL1R (B) were labeled with [myo-3H]inositol for IP3 assay. The levels of IP3 formation that reflect the activities of PLC were determined 30 min after individual or costimulation with specific agonists as indicated. Values shown represent the mean ± S.E. from three separate experiments. * treatment of bombesin in the absence or presence of dopamine significantly enhanced the IP3 levels compared with the corresponding basal.

![Fig. 6.](image)

Sp-cAMPs selectively suppressed the JNK activation mediated by GRPR rather than ORL1R. COS-7 cells coexpressing JNK-HA and D1R with either GRPR (A) or ORL1R (B) were treated with Sp-cAMPs (Sp, 100 μM) and the indicated agonists separately or simultaneously. The JNK activities were determined 30 min after separate or combinatory treatment as indicated. Values shown represent the mean ± S.E. from three independent experiments. * individual or combinatory treatment resulted in significant activation of JNK activity. #, cotreatment with Sp-cAMPs (100 μM) significantly inhibited the bombesin-induced JNK activation (Bonferroni paired t test, P < 0.05).
hypothesis, COS-7 cells coexpressing Gs-coupled GRPR and Gi-coupled D1R were pretreated with BAPTA-AM to deplete intracellular Ca$^{2+}$, followed by costimulation with bombesin and dopamine. COS-7 cells coexpressing Gs-coupled ORL1R and Gi-coupled D1R and costimulated with nociceptin and dopamine were used as a control for comparison. Indeed, our results showed that disruption of Ca$^{2+}$ signaling by BAPTA-AM attenuated the GRPR/D1R-induced JNK activity to a level similar to that of the ORL1R/D1R-mediated response (Fig. 8). This finding further demonstrates the importance of Ca$^{2+}$ mobilization for the GRPR/D1R-induced JNK response, whereas a cAMP signal (initiated from Gs and further potentiated by Ga) exhibits an inhibitory effect on the activity of the JNK cascade (Fig. 9B).

**Discussion**

Receptors coupled to Gs have been suggested to use cAMP to activate JNK. In the present report, we provide evidence that Gs-linked receptors are also capable of stimulating this kinase via an alternative pathway, in which G$\beta\gamma$ subunits serve as the primary player in the signal transduction. Because the various studies were performed in different cellular models, we cannot rule out the possibility that such differential dependence is a function of cell-type specificity. Irrespective of whether the Gs-mediated JNK activation is processed in a cAMP-dependent (Zhen et al., 1998) or -independent manner (as shown in this report), it seems that small GTPases serve as common intermediates for both signaling models.

The activities of small GTPases are regulated by GEFs. cAMP-responsive GEFs that can trigger ERK activation have been proposed (Laroche-Joubert et al., 2002), but the corresponding candidates for JNK regulation remain unclear. An exchange protein activated by cAMP (Epac) has been suggested as a cAMP-responsive GEF in rat renal collecting duct tubule cells for the activation of the small GTPase, Rap, which activates the ERK (Laroche-Joubert et al., 2002) rather than the JNK cascade (Mochizuki et al., 2000). However, selective activations of PKA and Epac in various cell lines (including Chinese hamster ovary, PC12, and human embryonic kidney 293 cells) revealed that the former, but not the latter, contributes to the cAMP-dependent ERK activation (Enserink et al., 2002). A recent study even proposed that Epac is capable of activating the JNK cascade in a small GTPase-independent manner (Hochbaum et al., 2003). These studies support opposing ideas, and it remains unclear as to the identities of GEFs, which are responsible for cAMP-mediated JNK activation.

![Fig. 7.](image1) Sp-cAMPs suppressed the bombesin-induced JNK activation in a dose-dependent manner. COS-7 cells coexpressing JNK-HA, D1R, and GRPR were stimulated by bombesin (100 nM) accompanied with increasing concentrations of Sp-cAMPs (0–1000 μM). The JNK activities were determined 30 min after individual or combinatory treatment as indicated. Values shown represent the mean ± S.E. from three separate experiments with the bombesin-induced JNK activity defined as 100%. #, cotreatment with Sp-cAMPs significantly inhibited the bombesin-induced JNK activity (Bonferroni paired t test, $P < 0.05$).

![Fig. 8.](image2) Disruption of Ca$^{2+}$ signaling by BAPTA-AM suppressed the D1R/GRPR-mediated JNK activity to the D1R/ORL1R-induced level. COS-7 cells coexpressing JNK-HA and D1R with either GRPR or ORL1R were pretreated in the absence (control) or presence of BAPTA-AM (10 μM, 30 min) followed by the addition of their specific agonists. The JNK activities were determined 30 min after the combinatory drug treatment. Values shown represent the mean ± S.E. from three separate experiments. *, coadministration of agonists resulted in significant activation of JNK activities compared with their corresponding basal. #, pretreatment with BAPTA-AM significantly suppressed the JNK activity induced by costimulation with bombesin and dopamine (Bonferroni paired t test, $P < 0.05$).
a Rac-dependent manner (Kiyono et al., 2000). In COS-7 cells, both G<sub>1</sub>- and G<sub>q</sub>-coupled receptors trigger JNK activation via a G<sub>βγ</sub>/Src-dependent mechanism, which can be suppressed by dominant-negative mutants of both Sos (i.e., Son of Sevenless) and small GTPases (Chan and Wong, 2004b; Kam et al., 2004). These findings clearly support the involvement of GEFs and small GTPases in the activation of JNK by GPCRs. In addition to Src-like kinases, PI3K acts as a downstream effector for G<sub>βγ</sub> (Lopez-Ilasaca et al., 1998), and its phospholipid products are capable of regulating the binding of certain GEFs to Rac (Das et al., 2000). EGFR transactivation has been proposed as a possible route that links GPCR signaling to the activation of MAPK subgroups (Luttrell et al., 1997). However, our results clearly demonstrated that G<sub>q</sub>-coupled receptors (as least for the D<sub>1</sub>R and LHR), similar to other receptors linked to G<sub>i</sub> and G<sub>q</sub> (Chan and Wong, 2004b; Kam et al., 2004), used Src-like kinases rather than PI3K or EGFR as the primary intermediate to stimulate JNK activity in COS-7 cells. It should be noted that further investigations on other G<sub>q</sub>-coupled receptors are required to establish a more definitive conclusion on the G<sub>q</sub>-induced activation of JNK.

Investigations on receptors with specific G protein-coupling preferences allow us to understand how different G proteins use various intermediates to trigger a biological response. However, in a physiological environment, cells are likely to have their receptors costimulated by different arrays of extracellular stimulus, and the resulting outcomes are determined by the integration of signals that occur inside the cells. There is increasing evidence to demonstrate the existence of signaling “cross-talk” between GPCRs of different coupling specificities (Selbie and Hill, 1998; Hur and Kim, 2002). By expressing different GPCRs in COS-7 cells followed by stimulation with specific agonists individually or simultaneously, complex patterns are observed for the regulation of cAMP and IP<sub>3</sub> levels, as well as for JNK activities.

Costimulation of G<sub>q</sub>-coupled D<sub>1</sub>R and G<sub>q</sub>-coupled ORL<sub>1</sub>R resulted in an elevated cAMP level which was lower than that of the D<sub>1</sub>R response, but no significant enhancement of IP<sub>3</sub> level could be observed (Fig. 5). As illustrated in the present report, cAMP is not as effective as G<sub>βγ</sub> subunits for the triggering of JNK activation in COS-7 cells. Hence, the G<sub>βγ</sub> subunits released from G<sub>q</sub> and G<sub>i</sub> may cooperate with each other, using a G<sub>βγ</sub>/Src-dependent pathway to mediate the JNK activation in a roughly additive manner. The reason for higher JNK-activating capability associated with G<sub>q</sub> compared with G<sub>i</sub> is currently unknown, but it should be borne in mind that the G<sub>α</sub> subunits of different G protein members may preferably associate with specific isoforms of G<sub>βγ</sub> subunits (Albert and Robillard, 2002), which may in turn mediate the downstream signaling in a similar but not identical manner. On the other hand, the total amount of G<sub>βγ</sub> subunits releasable from G<sub>q</sub>/G<sub>i</sub> (with five subtypes) is expected to be greater than those from G<sub>q</sub>/G<sub>i</sub>. Hence, G<sub>q</sub> may weakly augment the G<sub>i</sub>-mediated JNK activation by contributing additional G<sub>βγ</sub> subunits to the pathway (Fig. 9A).

The G<sub>q</sub> signal neither potentiates nor inhibits the G<sub>q</sub>-mediated IP<sub>3</sub> formation in COS-7 cells. In contrast, G<sub>q</sub>-induced cAMP elevation is synergistically potentiated by the Ca<sup>2+</sup>-signal from G<sub>q</sub>, indicating that the Ca<sup>2+</sup>-responsive adenyl cyclase isoforms (e.g., type I, V, and VI) may be predominantly expressed in the cells (Wayman et al., 1994). It is interesting that G<sub>i</sub>-induced cAMP signaling is associated with an inhibitory function on the Ca<sup>2+</sup>-dependent JNK pathway. Such an effect may be magnified in the presence of G<sub>q</sub>-mediated synergistic cAMP formation, which in turn diminishes the cooperative effect between Ca<sup>2+</sup> and G<sub>βγ</sub> of G<sub>q</sub> (Chan and Wong, 2004b), resulting in a decreased JNK activation compared with the normal G<sub>q</sub>-mediated response.
This idea is supported by our previous finding that activation of vasopressin V2 receptor (with dual coupling specificities toward Gs and Gi) triggers JNK activity with a magnitude higher than the Gs but lower than the Gi-mediated activity (Chan and Wong, 2004a). In fact, Gi-mediated JNK pathway may involve the Ca2+-responsive focal adhesion kinase family (e.g., Pyk2) as important intermediates, and the possible cAMP-mediated inhibition toward this kinase signaling (Li et al., 1997) supports our mechanistic model for the Gi/Gs-integrated JNK activity.

One might argue that the decreased GRPR-mediated JNK activation could be caused by receptor desensitization induced by D1R signaling components (e.g., the cAMP-dependent protein kinase). It should be noted that the elevated IP3 formation triggered by GRPR was not affected by simultaneous activation of D1R (Fig. 5A). Hence, it is unlikely that D1R signaling suppresses the GRPR-mediated JNK activation by down-regulating the components in the GRPR/Gq/phospholipase C pathway. Although the present study did not provide any information regarding the possible occurrence of receptor dimerization, the suppression on GRPR-mediated JNK activation by D1R signaling (Fig. 3B) or direct administration of Sp-cAMPs (Fig. 6A) implied that, irrespective of possible receptor dimerization (between GRPR and D1R), the Gs/cAMP signal definitely possesses an inhibitory effect on the Gq/Ca2+-mediated JNK activation in COS-7 cells (Chan and Wong, 2004a).

We have demonstrated previously that GPCRs of different coupling specificities have differential abilities to activate JNK (i.e., Gs >> Gi >> Gq), and signals from receptor tyrosine kinases (e.g., EGF receptor) selectively augment the Gi-mediated stimulation of JNK activity in COS-7 cells (Chan and Wong, 2004a). In this report, we further illustrate that modulation of JNK activity also exists in the integration of GPCR signals. Because the JNK activation induced by Gi2-coupled receptors (including GRPR, bradykinin BK2, muscarinic acetylcholine M4, and histamine H1 receptors) in COS-7 cells are Ca2+-dependent (Chan and Wong, 2004b), and cAMP serves as an effective inhibitor for the Ca2+-induced JNK activity in the same cells (Chan and Wong, 2004a), it is possible that the JNK activation triggered by other Gi2-coupled receptors is subject to similar inhibitory regulation by the Gq/cAMP signal. The JNK activity induced by Gi2-coupled receptors was diminished by coactivation of Gi2-coupled receptors, resulting in a kinase activity lower than the Gi2 but higher than the Gs- and Gi2-mediated responses. On the other hand, the Gi2-induced JNK activity was weakly enhanced upon costimulation of Gi2-coupled receptor. Taking all of these findings together, a graded activation profile of JNK could be achieved by Gs-coupled receptor signaling and extracellular signal-regulated protein kinase in adenovirus receptor-mediated interleukin-8 production in human mast cells. Mol Pharmacol 55:726–734.


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