

**G_q-mediated activation of JNK by the GRP receptor is
inhibited upon co-stimulation of the G_s-coupled dopamine
D₁ receptor in Cos-7 cells**

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Running title: G_s /cAMP inhibits G_q -mediated JNK activation in Cos-7 cells

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Abbreviations: GRPR, gastrin-releasing peptide-preferring bombesin receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ORL₁R, opioid receptor-like receptor; PI3K, phosphoinositide-3-kinase.

ABSTRACT

G protein-coupled receptors (GPCRs) of G_i or G_q coupling specificity are effectively linked to activation of the JNK cascade. However, little is known with regard to the regulation of JNK by G_s -coupled receptors. In this report, we utilized Cos-7 cells transfected with the dopamine D_1 receptor (D_1R) to illustrate the signaling mechanism for G_s -mediated JNK activation. Stimulation of D_1R triggered a weak but significant elevation of JNK activity in a time- and dose-dependent manner. This D_1R -mediated JNK activation required the participation of $G\beta\gamma$, Src-like kinases and small GTPases, while disruptions of cAMP-, PI3K-, and EGFR-mediated signaling had no effect. Co-stimulation of D_1R with GPCRs of other coupling specificities resulted in differential activation profiles of JNK. Activation of G_s -coupled D_1R weakly potentiated the JNK activation induced by the G_i -coupled opioid receptor-like receptor (ORL_1R), but exhibited a significant inhibitory effect on the kinase activity triggered by the G_q -coupled gastrin-releasing peptide-preferring bombesin receptor (GRPR). Administration of Sp-cAMPS (a cAMP analogue which mimics the G_s /cAMP signal) also suppressed the JNK activation mediated by G_q -coupled GRPR, as well as the Ca^{2+} -induced kinase activation upon thapsigargin treatment. Moreover, the Ca^{2+} signal from GRPR synergistically potentiated the D_1R -triggered cAMP elevation, when the two receptors were simultaneously stimulated. Taken together, our results demonstrated that stimulation of G_s -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.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are expressed in nearly all eukaryotic cells, and the basic assembly of MAPK pathways is a three-component module conserved from yeast to human (i.e. MAPK kinase kinase \rightarrow MAPK kinase \rightarrow MAPK). At least three subtypes of MAPK have been identified so far, they are extracellular signal-regulated kinase (ERK), 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, differentiation, survival, to apoptosis (Dunn *et al.*, 2002). It is believed that JNK may exhibit its multi-functional 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 G_i and G_q families of G proteins, are efficiently linked to the activation of JNK. G_i -coupled receptors mainly require a $G\beta\gamma$ /Src-dependent mechanism to stimulate the JNK cascade (Chan and Wong, 2004a; Kam *et al.*, 2004), while G_q -coupled receptors utilize both $G\beta\gamma$ /Src and Ca^{2+} 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 trans-activation of epidermal growth factor receptors (EGFR) have been proposed as alternative routes for the $G\beta\gamma$ -mediated pathway (Lopez-Illasaca *et al.*, 1998; Murga *et al.*, 2000; Pierce *et al.*, 2001). Despite the possible differential involvements of signaling intermediates, $G\beta\gamma$ seems to play an important role for both G_i and G_q -mediated JNK activation in response to stimulation of GPCRs.

Receptors coupled to the G_s family of G proteins are characterized by their abilities to trigger adenylyl cyclase-mediated cAMP formation (Balmforth *et al.*, 1986). Among all GPCRs which show coupling preferences toward a particular G protein family, much less is known with regard to the stimulation of JNK by G_s-coupled receptor. It has been demonstrated that activation of dopamine D₁ receptors in SK-N-MC 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 β₂-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_s-mediated JNK stimulation (Yamauchi *et al.*, 2001). In contrast, numerous studies have demonstrated that G_s-coupled receptors generally lack the ability to stimulate JNK. For instance, activation of G_s-coupled adenosine A_{2A} receptor in HMC-1 human mast cells does not enhance the JNK activity (Feoktistov *et al.*, 1999). Likewise, studies of Chinese hamster ovary (CHO) cells over-expressing G_s-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_s-coupled receptors to stimulate JNK, cAMP has been suggested as an activator for JNK activity. Administration of cAMP analogues (e.g. 8-Br-cAMP) and adenylyl cyclase stimulants (e.g. forskolin) have 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_s-coupled receptors, or administration of cAMP-elevating agents, may suppress the kinase activation triggered by GPCRs of other coupling specificities.

We have previously demonstrated that transfected Cos-7 cells transiently expressing GPCRs are useful cellular models to study the activation of JNK mediated by G_i- and G_q-coupled receptors (Chan and Wong, 2000; Chan *et al.*, 2002; Chan and Wong, 2004a), with the experimental results highly consistent with those obtained from endogenous cellular systems (Kam *et al.*, 2003; Kam *et al.*, 2004; Chan and Wong, 2004b). In this report, we utilized Cos-7 cells transfected with G_s-coupled dopamine D₁ receptor (D₁R) to illustrate the signaling mechanism for JNK activation, and further investigated the effects of co-stimulation of G_s-coupled receptor with G_i- or G_q-coupled receptors on the JNK activity. Our results suggest that stimulation of G_s-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_i- and G_q-coupled receptors.

MATERIALS AND METHODS

Materials. The cDNAs encoding the GRPR and ORL₁R were kindly provided by Dr. Jim Battey (National Institutes of Health, Rockville) and Dr. Gang Pei (Shanghai Institutes for Biological Sciences, Shanghai), respectively. The plasmid encoding D₁R 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), RhoT19N and Cdc42T17N were provided by Dr. Marc Symons (Picower Institute for Medical Research, New York). The plasmid of HA-tagged JNK was donated by Dr. Tatyana Voyno-Yasenetskaya (University of Illinois, Chicago). [γ -³²P]ATP was purchased from DuPont NEN (Boston, MA). PTX and 12CA5 (Anti-HA) antibody were purchased from List Biological Laboratories (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 PLUSTM were obtained from Invitrogen (Carlsbad, CA). Bombesin, dopamine and nociceptin were purchased from Sigma (St. Louis, MO). Thapsigargin, BAPTA-AM, AG1478, radicicol, 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 μ g/ml streptomycin, and were maintained at 37°C in an environment of 5% CO₂. Cos-7 cells were transferred to 6-well plates at 4x10⁵ cells/well (for JNK assay) or to 12-well plates at 1.5x10⁵ cells/well (for AC and PLC assays). Transfection was performed by means of Lipofectamine PLUSTM reagents following the supplier's instructions.

***In vitro* JNK assay.** 36 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 terminated by washing the cells with phosphate-buffered saline (PBS), followed by the addition of 500 μ l of ice-cold detergent-containing lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM NaP_2O_7 , 1% Triton X-100, 1 mM DTT, 200 μ M Na_3VO_4 , 100 μ M PMSF, 2 μ g/ml leupeptin, 4 μ g/ml aprotinin and 0.7 μ g/ml pepstatin). Lysates obtained were subjected to JNK assay as described previously (Chan and Wong, 2000). 50 μ l of each supernatant was used for the detection of JNK-HA expression, and the remaining (450 μ l) was incubated for 1 hour at 4°C with 12CA5 (Anti-HA) antibody (2 μ g/sample), followed by incubation with 30 μ l 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 $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, 1 mM EGTA, 1 mM DTT, 200 μ M Na_3VO_4). Washed immunoprecipitates were resuspended in 40 μ l of kinase assay buffer containing 5 μ g of GST-c-Jun per reaction, and the kinase reactions were initiated by the addition of 10 μ l of ATP buffer (50 μ M ATP containing 2 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ per sample). After 30 min incubation at 30°C with occasional shaking, the reactions were terminated by 10 μ l of 6X 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 (Molecular Dynamics 445 SI).

Phospholipase C (PLC) assay. One day after transfection, Cos-7 cells were labeled for 18 h with 0.75 ml of inositol-free DMEM containing $[\text{}^3\text{H}]\text{-myo-inositol}$ (5 μ Ci/ml) and 10% FCS (v/v), followed by serum starvation for 18 h. The cells were then pre-treated 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 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 (AC) assay. Transfected Cos-7 cells were labeled with 2 μ Ci/ml of [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 of HEPES and 1 mM of 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 (TCA) 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 previously demonstrated that Cos-7 cells transiently expressing D₁R serve as a reliable model for studying G_s-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 μ M of dopamine (Fig. 1B). To examine whether this induced kinase activation was contributed by the G_s/cAMP signaling, the cells were pretreated with Rp-cAMPS, a cell-permeable cAMP analogue with inhibitory effects on cAMP/PKA-regulated cellular events. Our results showed that Rp-cAMPS (100 μ M) had no significant inhibitory effect on the D₁R-induced JNK activity (Fig. 1C). Further investigation on the G_s-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 μ M) 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 G_i- and G_q-coupled receptors appears to depend on G $\beta\gamma$ subunits (Chan and Wong, 2004a, 2004b). Such a response can be suppressed by transducin which acts as an effective scavenger to remove free G $\beta\gamma$ subunits released upon G protein activation. For Cos-7 cells expressing G_s-coupled D₁R, the dopamine-induced JNK activation was almost completely inhibited when transducin was co-expressed in the cells (Fig. 2A). This is consistent with a previous report

wherein $G\beta\gamma$ subunits are better activators than the α -subunit of G_s ($G\alpha_s$) in terms of JNK activation (Coso *et al.*, 1996). In order to reveal the identities of $G\beta\gamma$ -regulated intermediates for the D_1R -mediated JNK activation, target-specific inhibitors (radicol and PP1 for Src-like kinases, wortmannin for PI3K isoforms, and AG1478 for EGFR) were employed. Pretreatment of radicol (Fig. 2A) and PP1 (data not shown) significantly suppressed the D_1R -mediated JNK activation, while 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 are usually performed by expression of the corresponding dominant negative mutants of these small GTPases (i.e. RasS17N, RacT17N, RhoT19N and Cdc42T17N). When D_1R was co-expressed 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 G_s -coupled LHR was also characterized by dependencies on $G\beta\gamma$, 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_i -coupled ORL_1R and G_q -coupled $GRPR$. The preceding experiments demonstrated that G_s -coupled D_1R activated JNK in a $G\beta\gamma$ -, 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_i -coupled ORL_1R (Chan and Wong, 2000) and G_q -coupled $GRPR$ (Chan and Wong, 2004b). In order to investigate the integrated JNK activities upon GPCR co-activation, we co-transfected G_s -coupled D_1R with either G_i -coupled ORL_1R (Fig. 3A), or G_q -coupled $GRPR$ (Fig. 3B) in Cos-7 cells, followed by individual or co-administration of corresponding agonists. For cells co-expressing D_1R and ORL_1R , individual

treatment with appropriate agonists (dopamine for D₁R and nociceptin for ORL₁R) triggered JNK activation to ~1.5 fold and ~2.0 fold, respectively (Fig. 3A), as 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 D₁R, and with our previous report for ORL₁R (Chan and Wong, 2000), using Cos-7 cells transiently expressing either one of the receptors. Co-stimulation of both D₁R and ORL₁R 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 D₁R and ORL₁R utilize a similar mechanistic pathway (Gβγ → Src-like kinase → small GTPase → JNK cascade), D₁R remained capable of complementing the kinase response triggered by ORL₁R. To reveal if G_s-coupled D₁R also exhibits a similar effect on the JNK activity stimulated by G_q-coupled GRPR, Cos-7 cells were co-transfected with these two receptors. Activation of D₁R by dopamine retained the ability to activate JNK weakly, and the GRPR agonist (i.e. bombesin) was still capable of triggering a 6-7 fold kinase activity (Fig. 3B), similar to our results in the previous report (Chan and Wong, 2004b). Unexpectedly, when both D₁R and GRPR were co-stimulated in the same cells, the induced JNK activation was significantly decreased as compared with the GRPR-mediated response (Fig. 3B). These results suggested that even though the G_s-coupled D₁R 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 D₁R-mediated adenylyl cyclase activation by G_i-coupled ORL₁R and G_q-coupled GRPR. The differential regulatory effects of D₁R on the ORL₁R- and GRPR-induced JNK activity (Fig. 3) implied that, although G_s-, G_i-, and G_q-coupled receptors utilize a common Gβγ-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 co-expressing G_s -coupled D_1R and G_i -coupled ORL_1R , administration of dopamine triggered an elevated cAMP level, while nociceptin treatment induced no observable changes in cAMP production (Fig. 4A). When both of these two agonists were co-administered, a net increase of cAMP formation was produced, which was associated with a significant inhibition as compared to the dopamine-induced activity (Fig. 4A). This result agreed with the classical G protein signaling model that the inhibitory G_i signal opposed the stimulatory G_s signal on the adenylyl cyclase-mediated cAMP formation.

Similarly, when Cos-7 cells co-expressing 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, while bombesin did not significantly stimulate this activity (Fig. 4B). However, when dopamine and bombesin were co-administered, the induced cAMP production was nearly doubled as compared to the dopamine effect (Fig. 4B). This indicated that the G_q signal from GRPR synergizes with the G_s -mediated adenylyl cyclase activation by D_1R . To investigate the mechanism which gave rise to this synergistic response, a series of experiments was performed by target-specific inhibition on the G_q signaling, or by testing the $G\beta\gamma$ -dependency of adenylyl cyclase activation. For cells co-transfected with transducin, removal of $G\beta\gamma$ subunits (released from G_s and G_q) was incapable of eliminating the synergistic response upon co-stimulation of D_1R 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 Ca^{2+} 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 G_q -induced Ca^{2+} activity is important for the GRPR-mediated potentiation of the D_1R -triggered cAMP elevation. In order to provide direct evidence for the synergism of G_s -mediated cAMP formation in response to the elevated Ca^{2+} activity, Cos-7 cells expressing D_1R alone were stimulated with dopamine and thapsigargin (an extensively used agent for elevating intracellular Ca^{2+} level) separately or simultaneously. Again, dopamine treatment significantly enhanced the cAMP level but thapsigargin failed to do so, while their co-application generated synergistic adenylyl cyclase activation (Fig. 4F). These results showed that the G_q -coupled GRPR may utilize a Ca^{2+} signal to potentiate the adenylyl cyclase activity induced by G_s -coupled D_1R .

Co-stimulation of G_s -coupled D_1R with either G_q -coupled GRPR or G_i -coupled ORL_1R had no effect on the PLC activity. G_q -coupled receptors are known to utilize both $G\beta\gamma$ - as well as PLC-dependent pathways to regulate the JNK activity (Chan and Wong, 2004b). Thus, we examined if signal integration might have occurred along the G_q /PLC pathway. In Cos-7 cells co-expressing G_q -coupled GRPR and G_s -coupled D_1R , treatment with bombesin stimulated IP_3 formation, while activation of D_1R by dopamine neither stimulated PLC, nor potentiated the GRPR-triggered response (Fig. 5A). Since the activities of PLC β -isoforms may be potentiated by $G\beta\gamma$ subunits released upon G_i activation (Chan *et al.*, 2000), a similar assay was hence performed with Cos-7 cells co-expressing G_s -coupled D_1R and G_i -coupled ORL_1R to determine whether co-operative signaling between G_s and G_i is capable of elevating the IP_3 formation. However, irrespective of whether these cells were treated with dopamine and nociceptin individually or simultaneously, no significant enhancement of IP_3 formation was observed (Fig. 5B).

cAMP serves as a suppressor of G_q-mediated JNK activation in Cos-7 cells. The preceding experiments demonstrated that co-stimulation 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 if the cAMP signal can suppress G_q-mediated JNK activation, Cos-7 cells were transfected with D₁R and GRPR and then treated with Sp-cAMPS (a cell-permeable analogue 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 G_i-coupled ORL₁R suggested that cAMP signaling specifically inhibited G_q- but not G_i-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 towards Sp-cAMPS (Fig. 7). In addition to the Gβγ-dependent pathway, G_q-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 G_s-mediated suppression of the G_q-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, co-treatment with increasing concentration of Sp-cAMPS resulted in a gradual decrease of kinase activities (Chan and Wong, 2004a). All these data support the idea that co-operation of G_s and G_q signaling may result in decreased G_q-mediated JNK activation, which is primarily caused by a suppressive effect of cAMP on the Ca²⁺ signaling component of the G_q-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, G_s-, G_i- and G_q-coupled receptors utilize the Gβγ-dependent pathway to regulate the activity of JNK cascade in Cos-7

cells, with the G_q -mediated response associated with an additional involvement of Ca^{2+} signaling. These characteristics imply that in the absence of the elevated Ca^{2+} signal upon G_q activation, co-stimulation of G_q and G_s should be similar to that of G_i and G_s , in terms of JNK activation mediated by $G\beta\gamma$ subunits from two different G protein sub-families. In order to examine this hypothesis, Cos-7 cells co-expressing G_q -coupled GRPR and G_s -coupled D_1R were pre-treated with BAPTA-AM to deplete intracellular Ca^{2+} , followed by co-stimulation with bombesin and dopamine. Cos-7 cells co-expressing G_i -coupled ORL_1R and G_s -coupled D_1R and co-stimulated with nociceptin and dopamine were employed as a control for comparison. Indeed, our results showed that disruption of Ca^{2+} signaling by BAPTA-AM attenuated the GRPR/ D_1R -induced JNK activity to a level similar to that of the ORL_1R / D_1R -mediated response (Fig. 8). This finding further demonstrates the importance of Ca^{2+} mobilization for the GRPR/ D_1R -induced JNK response, whereas a cAMP signal (initiated from G_s and further potentiated by G_q) exhibits an inhibitory effect on the activity of the JNK cascade (Fig. 9B).

DISCUSSION

Receptors coupled to G_s have been suggested to utilize cAMP to activate JNK. In the present report, we provide evidence that G_s-linked receptors are also capable of stimulating this kinase via an alternative pathway, in which Gβγ subunits serve as the primary player in the signal transduction. Since the various studies were performed in different cellular models, we cannot rule out the possibility that such differential dependency is a function of cell type specificity. Irrespective of whether the G_s-mediated JNK activation is processed in a cAMP-dependent (Zhen *et al.*, 1998) or cAMP-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 guanine nucleotide exchange factors (GEFs). cAMP-responsive GEFs which 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 CHO, PC12 and HEK293 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.

In contrast, GEFs which are responsive to $G\beta\gamma$ and the subsequent downstream Src-like kinases have been defined more clearly. The activity of Ras-GRF1 (a GEF for Ras and Rac) can be promoted through tyrosine phosphorylation by Src, which in turn leads to activation of JNK in a Rac-dependent manner (Kiyono *et al.*, 2000). In Cos-7 cells, both G_i - and G_q -coupled receptors trigger JNK activation via a $G\beta\gamma$ /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 $G\beta\gamma$ (Lopez-Illasaca *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 which links GPCR signaling to the activation of MAPK subgroups (Luttrell *et al.*, 1997). However, our results clearly demonstrated that G_s -coupled receptors (as least for the D_1R and LHR), similar to other receptors linked to G_i and G_q (Chan and Wong, 2004b; Kam *et al.*, 2004), utilized 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_s -coupled receptors are required in order to establish a more definitive conclusion on the G_s -induced activation of JNK.

Investigations on receptors with specific G protein coupling preferences allow us to understand how different G proteins utilize various intermediates to trigger a biological response. However, in a physiological environment, cells are likely to have their receptors co-stimulated 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 IP₃ levels, as well as for JNK activities.

Co-stimulation of G_s-coupled D₁R and G_i-coupled ORL₁R resulted in an elevated cAMP level which was lower than that of the D₁R response, but no significant enhancement of IP₃ level could be observed (Fig. 5). As illustrated in the present report, cAMP is not as effective as Gβγ subunits for the triggering of JNK activation in Cos-7 cells. Hence, the Gβγ subunits released from G_s and G_i may co-operate with each other, utilizing a Gβγ/Src-dependent pathway to mediate the JNK activation in a roughly additive manner. The reason for higher JNK activating capability associated with G_i as compared to G_s is currently unknown, but it should be borne in mind that the Gα subunits of different G protein members may preferably associate with specific isoforms of Gβγ subunits (Albert and Robillard, 2002), which may in turn mediate the downstream signaling in a similar, but not identical manner. Alternatively, the total amount of Gβγ subunits releasable from G_i/G_o (with five subtypes) is expected to be greater than those from G_s. Hence, G_s may weakly augment the G_i-mediated JNK activation by contributing additional Gβγ subunits to the pathway (Fig. 9A).

The G_s signal neither potentiates nor inhibits the G_q-mediated IP₃ formation in Cos-7 cells. In contrast, G_s-induced cAMP elevation is synergistically potentiated by the Ca²⁺ signal from G_q, indicating that the Ca²⁺-responsive adenylyl cyclase isoforms (e.g. type I, V and VI) may be predominantly expressed in the cells (Wayman *et al.*, 1994). Interestingly, G_s-induced cAMP signaling is associated with an inhibitory function on the Ca²⁺-dependent JNK pathway. Such an effect may be magnified in the presence of Ca²⁺-mediated synergistic cAMP formation,

which in turn diminishes the co-operative effect between Ca^{2+} and $\text{G}\beta\gamma$ of G_q (Chan and Wong, 2004b), resulting in a decreased JNK activation as compared to the normal G_q -mediated response (Fig. 9B). This idea is supported by our previous finding that, activation of vasopressin V_2 receptor (with dual coupling specificities towards G_s and G_q) triggers JNK activity with a magnitude higher than the G_s -, but lower than the G_q -mediated activity (Chan and Wong, 2004a). In fact, G_q -mediated JNK pathway may involve the Ca^{2+} -responsive focal adhesion kinase family (e.g. Pyk2) as important intermediates, and the possible cAMP-mediated inhibition towards this kinase signaling (Li *et al.*, 1997) supports our mechanistic model for the G_q/G_s -integrated JNK activity.

One might argue that the decreased GRPR-mediated JNK activation could be due to receptor desensitization induced by D_1R signaling components (e.g. the cAMP-dependent protein kinase). It should be noted that the elevated IP_3 formation triggered by GRPR was not affected by simultaneous activation of D_1R (Fig. 5A). Hence, it is unlikely that D_1R signaling suppresses the GRPR-mediated JNK activation by down-regulating the components in the GRPR/ G_q /phospholipase C pathway. Although the present study did not provide any information about the possible occurrence of receptor dimerization, however, the suppression on GRPR-mediated JNK activation by D_1R signaling (Fig. 3B) or direct administration of Sp-cAMPS (Fig. 6A) implied that, irrespective of possible receptor dimerization (between GRPR and D_1R), the G_s/cAMP signal definitely possesses an inhibitory effect on the $\text{G}_q/\text{Ca}^{2+}$ -mediated JNK activation in Cos-7 cells (Chan and Wong, 2004a).

We have previously demonstrated that GPCRs of different coupling specificities have differential abilities to activate JNK (i.e. $\text{G}_q > \text{G}_i > \text{G}_s$), and signals from receptor tyrosine kinases (e.g. EGF receptor) selectively augment the G_i -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. Since the JNK activation induced by G_q -coupled receptors (including GRPR, bradykinin BK_2 , muscarinic acetylcholine M_1 and histamine H_1 receptors) in Cos-7 cells are Ca^{2+} -dependent (Chan and Wong, 2004b), and cAMP serves as an effective inhibitor for the Ca^{2+} -induced JNK activity in the same cells (Chan and Wong, 2004a), it is possible that the JNK activation triggered by other G_q -coupled receptors are subject to similar inhibitory regulation by the G_s /cAMP signal. The JNK activity induced by G_q -coupled receptors was diminished by co-activation of G_s -coupled receptors, resulting in a kinase activity lower than G_q , but higher than the G_i - and G_s -mediated responses. On the other hand, the G_i -induced JNK activity was weakly enhanced upon co-stimulation of G_s -coupled receptor. Taking all these findings together, a graded activation profile of JNK could be achieved by linking GPCRs of different coupling preferences to a signaling network, in which specific (e.g. cAMP from G_s and Ca^{2+} from G_q) as well as common (e.g. $G\beta\gamma$ /Src) signals from different G proteins can be integrated with each other to modulate the activities of the JNK cascade (Fig. 9).

The present report illustrates the “fine tuning” character of JNK activation in response to cross-communication between GPCR of different coupling specificities. In fact, the magnitudes of activity as well as the duration of MAPK activation are required to trigger a sequential cascade of transcription factor induction, and their subsequent gene transcriptional activities (Harada *et al.*, 2001). The resulting gene products may in turn, serve as critical factors which determine cell fate such as proliferation, differentiation or apoptosis (Kobayashi and Tsukamoto, 2001). Further investigation should be focused on suitable endogenous cellular systems in order to define the possible biological consequence of this integrated JNK activity.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Activation of JNK triggered by G_s-coupled receptors. Cos-7 cells were co-transfected with the cDNAs of JNK-HA, the G_s-coupled D₁R (A, B, C and E), or the G_s-coupled LHR (D). D₁R-expressing cells were stimulated with dopamine (10 μM) for increasing durations (A), or incubated with increasing concentrations of the agonist for 30 min (B). Cells expressing D₁R (C and E) or LHR (D) were also pretreated in the absence (control) or presence of Rp-cAMP (100 μM, 30 min) before stimulation with their agonists (10 μM dopamine for D₁R and 1 μg/ml hCG for LHR). JNK assay was performed as described in *Materials and Methods*. The values shown represent the mean ± S.E. from at least four separate experiments. *Activation of D₁R (C) or LHR (D) significantly activate the JNK activity as compared to the basal (Bonferroni paired *t*-test, P<0.05). (E) D₁R-induced CREB phosphorylation was inhibited by pre-treatment with Rp-cAMPS.

Figure 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 G_s-coupled D₁R (A and B). The cells were either co-expressing transducin (Gα_t) 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 in *Materials and Methods*. Values shown represent the mean ± S.E. from three to six separate experiments. *Administration of dopamine induced significant activation of JNK as compared to the corresponding basal. #Removal of Gβγ by transducin, pretreatment with radicicol, and

the presence of RasS17N, RacT17N and Cdc42T17N significantly inhibited the JNK activation as compared with the control group (Bonferroni paired *t*-test, $P < 0.05$).

Figure 3. Differential regulatory effects of G_s -coupled D_1R on the JNK activation triggered by G_i -coupled ORL_1R and G_q -coupled GRPR. Cos-7 cells co-expressing JNK-HA and D_1R with ORL_1R (A) or GRPR (B) were stimulated with their corresponding agonists (10 μM dopamine, DOP for D_1R , 100 nM nociceptin, OFQ for ORL_1R , and 100 nM bombesin, BBS for GRPR) separately or simultaneously. The JNK activities were determined at 30 min after individual or combinatory treatment of these agonists as indicated. Values shown represent the mean \pm S.E. from four separate experiments. *Individual or combinatory treatment significantly increased the JNK activities as compared to the basal. #Co-stimulation of D_1R significantly inhibited the GRPR-induced JNK activation (Bonferroni paired *t*-test, $P < 0.05$).

Figure 4. Modulation of D_1R -mediated adenylyl cyclase activation by G_i -coupled ORL_1R and G_q -coupled GRPR. Cos-7 cells co-expressing D_1R with either ORL_1R (A) or GRPR in the absence (B, D, E) or presence (C) of transducin ($G\alpha_t$) were labeled with [3H]adenine (A – E). These cells were then pretreated with (D and E) or without (A, B and C) calphostin C (Cal. C, 10 μM , 30 min) or BAPTA-AM (10 μM , 30 min) for the inhibition of PKC and Ca^{2+} signaling, respectively. Cos-7 cells expressing D_1R alone were also labeled with [3H]adenine for cAMP assay (F). The assays for cAMP elevation which reflect the activity of adenylyl cyclase was determined at 30 min after individual or co-stimulation with specific agonists and thapsigargin (Thap, 5 μM) as indicated. Values shown represent the mean \pm S.E. from three separate experiments. *Dopamine (DOP, 10 μM) treatment in the absence or presence of nociceptin (OFQ, 100 nM) significantly enhanced the cAMP level as

compared to the basal (A). *Treatment with dopamine in the absence or presence of bombesin (B – E) or thapsigargin (F) significantly enhanced the cAMP level as compared to the corresponding basal. **Co-treatment with bombesin (B, C and D) or thapsigargin (F) significantly increased the D₁R-induced cAMP elevation. #Co-stimulation of ORL₁R significantly inhibited the D₁R-induced cAMP elevation (Bonferroni paired *t*-test, P<0.05).

Figure 5. Co-stimulation of G_s-coupled D₁R with either G_q-coupled GRPR or G_i-coupled ORL₁R had no effect on PLC. Cos-7 cells co-expressing D₁R with either GRPR (A) or ORL₁R (B) were labeled with [³H]myo-inositol for IP₃ assay. The levels of IP₃ formation which reflect the activities of PLC were determined at 30 min after individual or co-stimulation 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 as compared to the corresponding basal.

Figure 6. Sp-cAMPS selectively suppressed the JNK activation mediated by GRPR rather than ORL₁R. Cos-7 cells co-expressing 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 at 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. #Co-treatment with Sp-cAMPS (100 μM) significantly inhibited the bombesin-induced JNK activation (Bonferroni paired *t*-test, P<0.05).

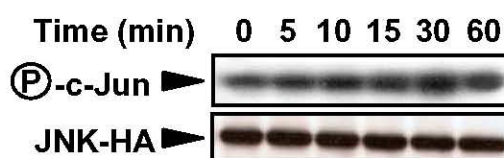
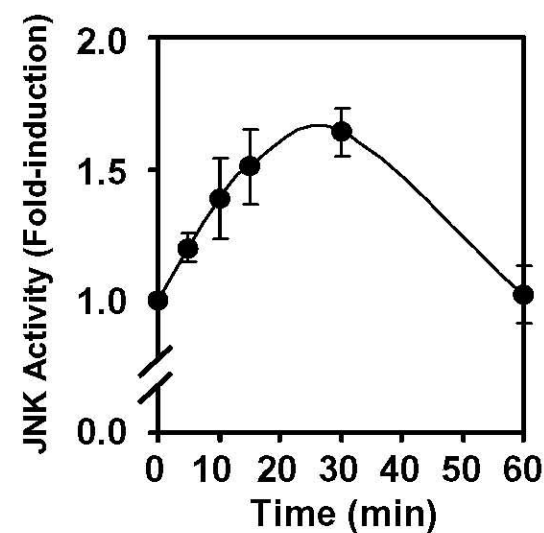
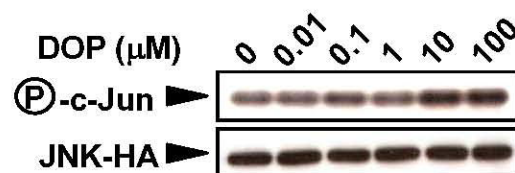
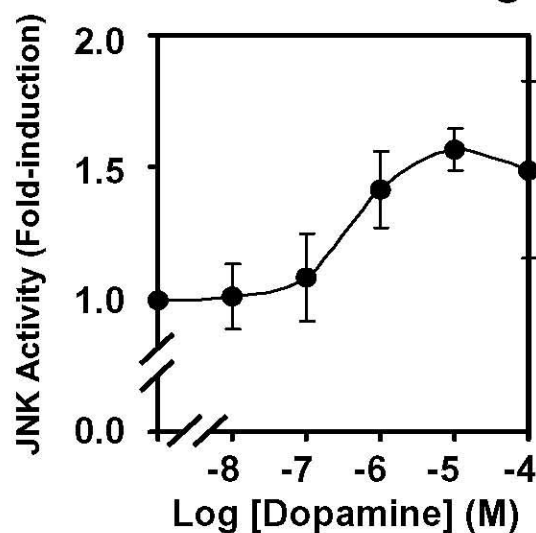
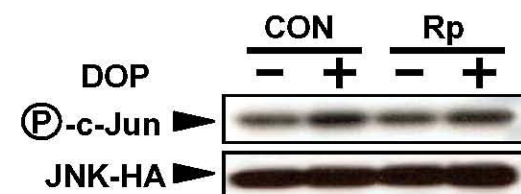
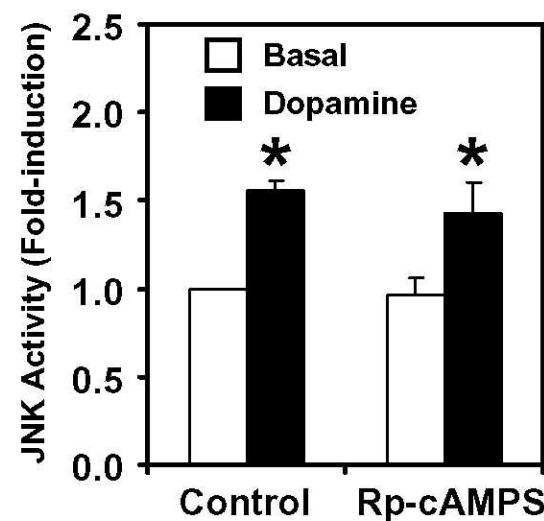
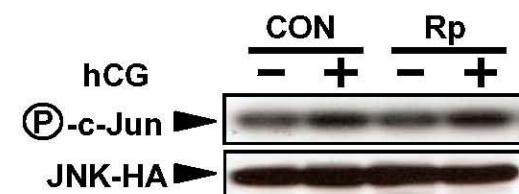
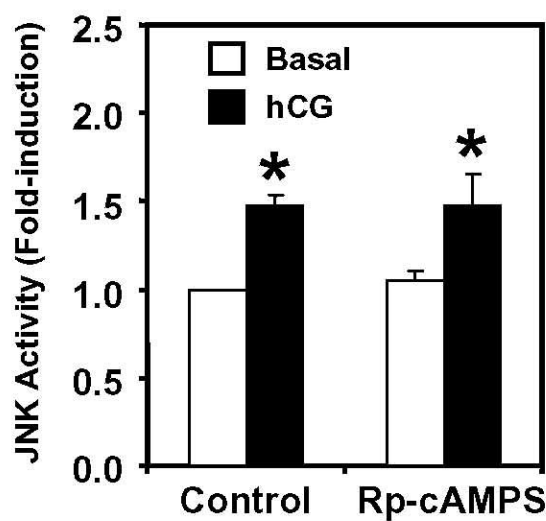
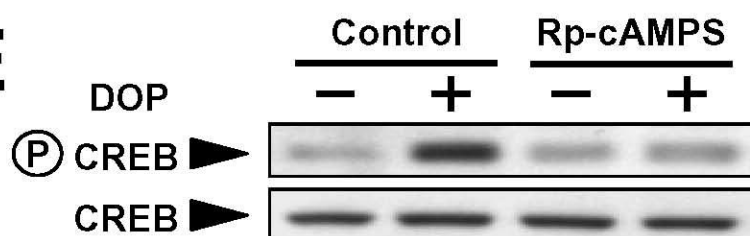
Figure 7. Sp-cAMPS suppressed the bombesin-induced JNK activation in a dose-dependent manner. Cos-7 cells co-expressing JNK-HA, D₁R and GRPR were stimulated by

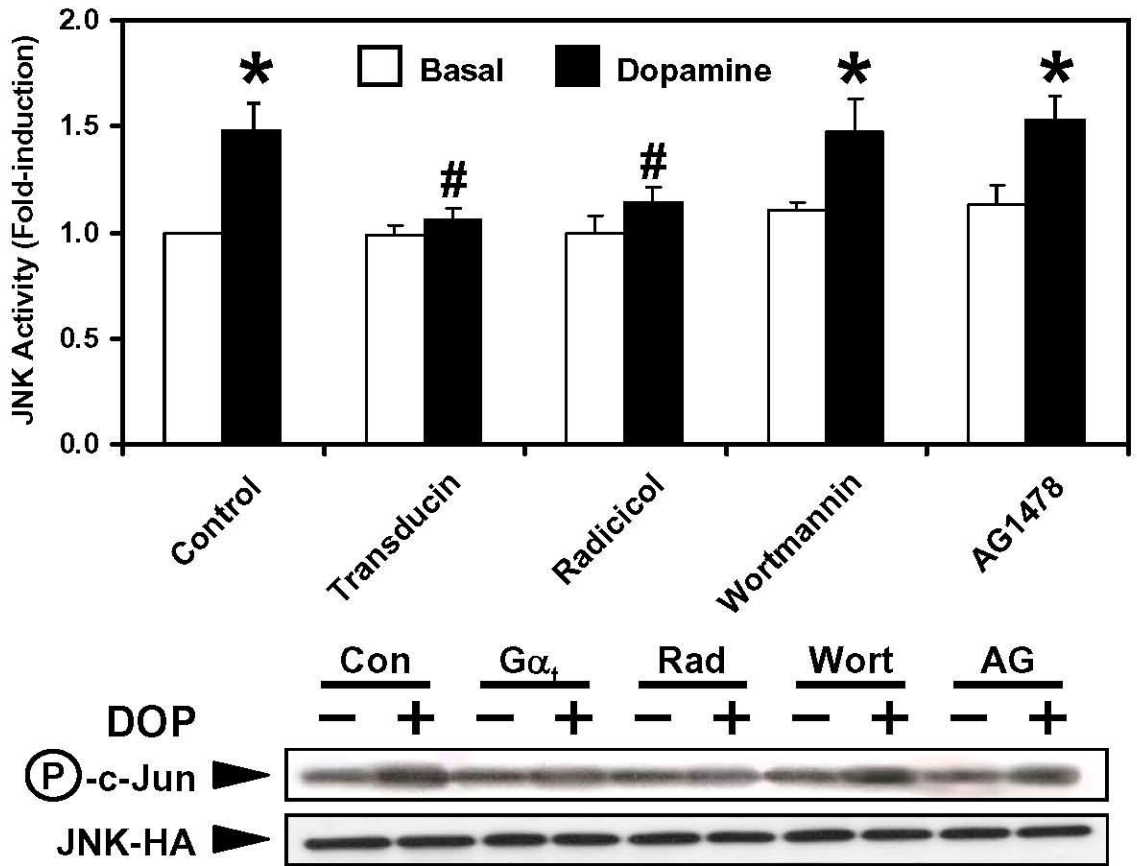
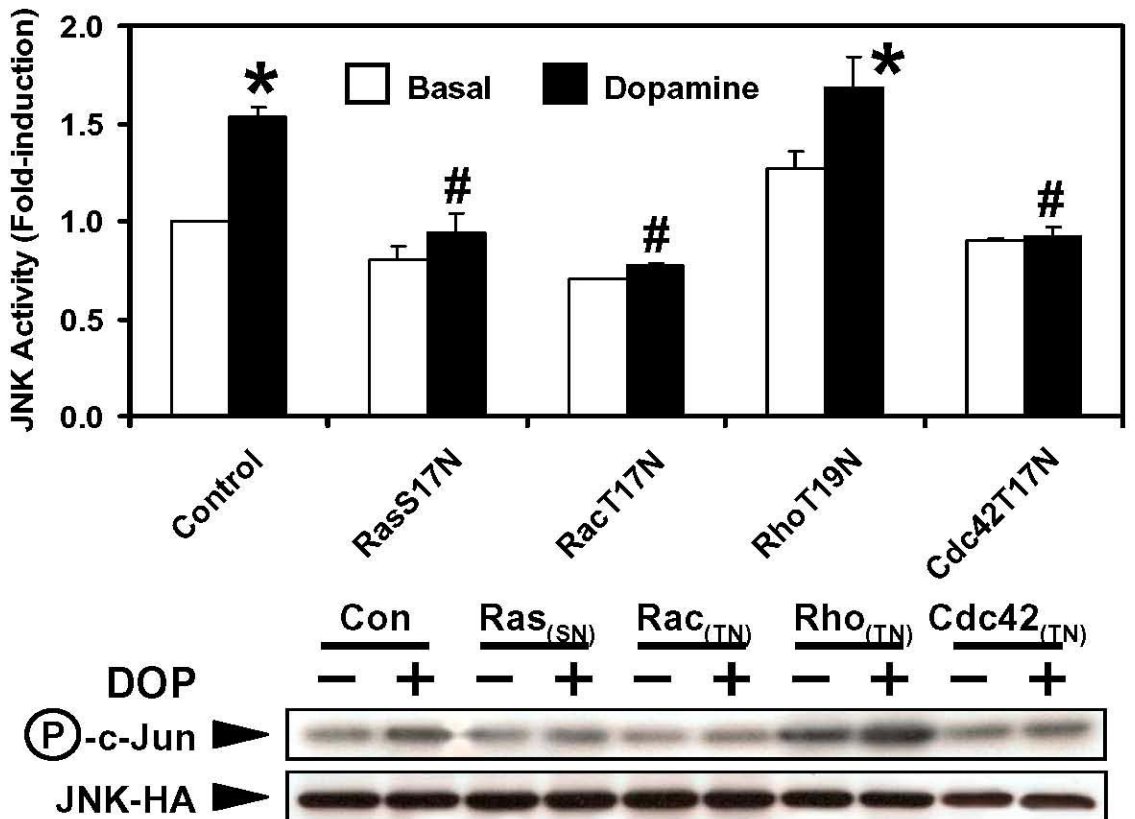
bombesin (100 nM) accompanied with increasing concentrations of Sp-cAMPS (0 – 1000 μ M). The JNK activities were determined at 30 min after individual or combinatory treatment as indicated. Values shown represent the mean \pm S.E. from three separate experiments, with the bombesin-induced JNK activation in the absence of Sp-cAMPS defined as 100%. [#]Co-treatment with Sp-cAMPS significantly inhibited the bombesin-induced JNK activation (Bonferroni paired *t*-test, *P*<0.05).

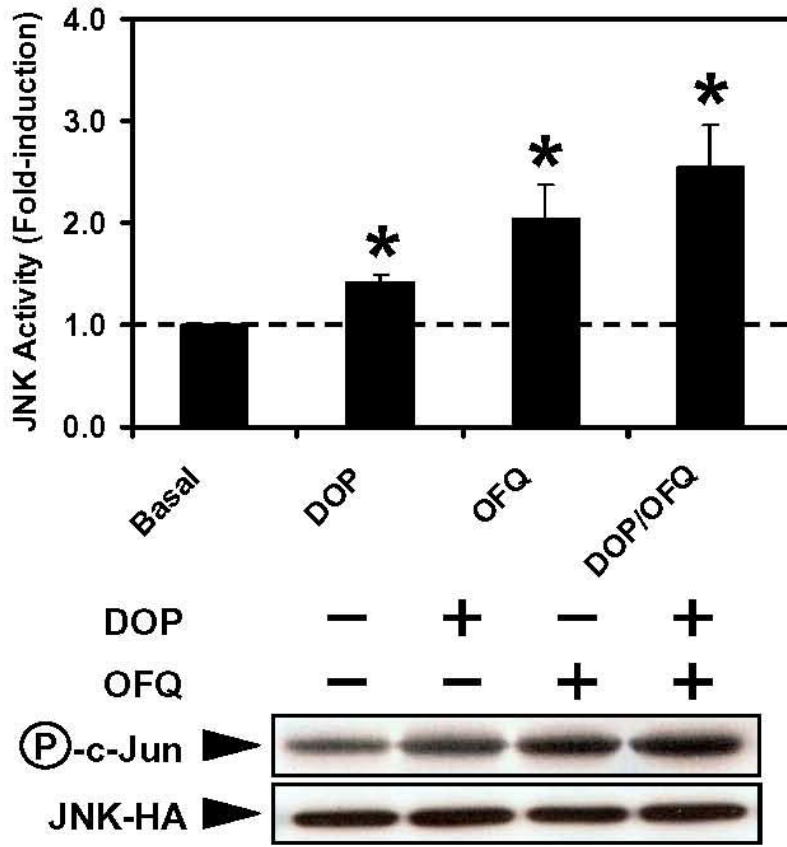
Figure 8. Disruption of Ca²⁺ signaling by BAPTA-AM suppressed the D₁R/GRPR-mediated JNK activity to the D₁R/ORL₁R-induced level. Cos-7 cells co-expressing JNK-HA and D₁R with either GRPR or ORL₁R were pretreated in the absence (control) or presence of BAPTA-AM (10 μ M, 30 min) followed by addition of their specific agonists. The JNK activities were determined at 30 min after the combinatory drug treatment. Values shown represent the mean \pm S.E. from three separate experiments. *Co-administration of agonists resulted in significant activation of JNK activities as compared to their corresponding basal. [#]Pre-treatment with BAPTA-AM significantly suppressed the JNK activity induced by co-stimulation with bombesin and dopamine (Bonferroni paired *t*-test, *P*<0.05).

Figure 9. Schematic diagrams for the regulatory effects of G_s on the G_i- and G_q-mediated JNK activation. (A) G $\beta\gamma$ subunits from G_s and G_i converge their signals at Src-like tyrosine kinases and subsequently trigger the activation of JNK cascade, while cAMP has no inhibitory effect on the G_i-mediated JNK activation. (B) G $\beta\gamma$ subunits from G_s and G_q converge their signals at Src-like kinases, and the G α_s -mediated cAMP elevation is synergistically potentiated in the presence of Ca²⁺-responsive adenylyl cyclase isoforms. The G $\beta\gamma$ /Src and Ca²⁺ signals co-operate with each other to regulate the JNK activation (Chan and

Wong, 2004b), while cAMP suppresses the induced kinase activation by interfering the Ca²⁺-mediated pathway.

A**B****C****D****E**

A**B**

A**B**