G_q-Mediated Activation of c-Jun N-Terminal Kinase by the Gastrin-Releasing Peptide-Preferring Bombesin Receptor Is Inhibited upon Costimulation of the G_s-Coupled Dopamine D_1 Receptor in COS-7 Cells

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ABSTRACT

G protein-coupled receptors (GPCRs) of G_i- 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 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, whereas disruptions of cAMP-, phosphoinositide-3-kinase-, and epidermal growth factor receptor-mediated signaling had no effect. Costimulation 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, but it 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-adenosine-3′,5′-cyclic monophosphorothioate triethylamine (a cAMP analog that 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 stimulated simultaneously. 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.

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 → MAPK kinase → MAPK). At least three subtypes of MAPK have been identified so far: 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, 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 G_i and G_q families of G proteins, are

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; GRPR, gastrin-releasing peptide-preferring bombesin receptor; JNK, c-Jun N-terminal kinase; ORL_1R, opioid receptor-like receptor; PI3K, phosphoinositide-3-kinase; GPCR, G protein-coupled receptor; D_1R, dopamine D_1 receptor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; HA, hemagglutinin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PLC, phospholipase C; LHR, lutropin hormone receptor; GEF, guanine nucleotide exchange factor; IP_3, inositol 1,4,5-trisphosphate; AG1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; Rp-cAMPs, adenosine 3′,5′-cyclic monophosphorothioate triethylamine, Rp-isomer; Sp-cAMPS, adenosine-3′,5′-cyclic monophosphorothioate triethylamine, Sp-isomer.

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), whereas G$_q$-coupled receptors use 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 transactivation of epidermal growth factor receptors (EGFRs) have been proposed as alternative routes for the G$\beta\gamma$-mediated pathway (Lopez-Ilasaca 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 that 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 D$_1$ 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 β_2-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 A2$_A$ 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$_s$-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 analogs (e.g., 8-bromo-adenosine-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$_s$-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$_i$- and G$_q$-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$_s$-coupled dopamine D$_1$ receptor (D$_1$R) to illustrate the signaling mechanism for JNK activation and further investigated the effects of costimulation 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 ORL$_1$R were kindly provided by Dr. Jim Battey (National Institutes of Health, Bethesda, MD) and Dr. Gang Pei (Shanghai Institutes for Biological Sciences, Shanghai, Hong Kong), respectively. The plasmid encoding D$_1$R 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 (Picower 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-^{32}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). Bombesin, dopamine, and nociceptin were purchased from Sigma-Chemical (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 μg/ml streptomycin and were maintained at 37°C in an environment of 5% CO$_2$. COS-7 cells were transferred to six-well plates at 4×10^5 cells/well (for JNK assay) or to 12-well plates at 1.5×10^5 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 NaP$_2$O$_7$, 1% Triton X-100, 1 mM dithiothreitol, 200 μM Na$_3$VO$_4$, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 0.7 μg/ml pepstatin). Lysates obtained were subjected to JNK assay as described previously (Chan and Wong, 2000). Fifty microliters of each supernatant was used for the detection of JNK-HA 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(C$_2$H$_3$O$_2$)$_2$, 1 mM EGTA, 1 mM dithiothreitol, and 200 μM Na$_3$VO$_4$]. 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-^{32}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*-^{3}H]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 [^{3}H]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-isobutyl-xanthine) 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 [^{3}H]cAMP were determined by sequential chromatography, as described previously (Chan and Wong, 2004a).

Results

Time/Dose-Dependencies of D_1R-Mediated JNK Activation. We have demonstrated previously that COS-7 cells transiently expressing D_1R serve as reliable models 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 D_1R transfectants with a fixed dose of dopamine for increasing durations, or with increasing doses for a fixed period. The D_1R-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 G_s/cAMP signaling, the cells were pretreated with Rp-cAMPs, a cell-permeable cAMP
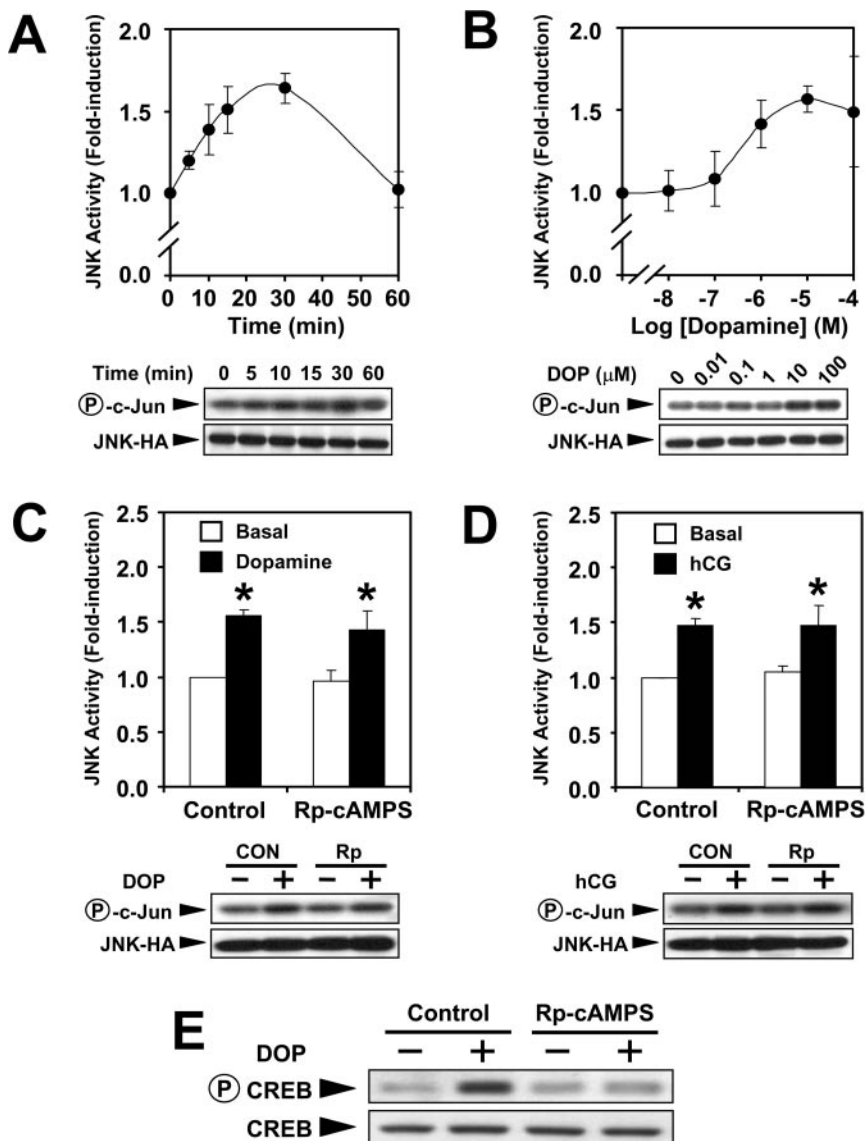

Fig. 1. Activation of JNK triggered by G_s-coupled receptors. COS-7 cells were cotransfected with the cDNAs of JNK-HA, the G_s-coupled D_1R (A, B, C, and E), or the G_s-coupled LHR (D). D_1R-expressing cells were stimulated with dopamine (10 μM) for increasing durations (A) or were incubated with increasing concentrations of the agonist for 30 min (B). Cells expressing D_1R (C and E) or LHR (D) were also pretreated in the absence (control) or presence of Rp-cAMPs (100 μM, 30 min) before stimulation with their agonists (10 μM dopamine for D_1R and 1 μg/ml human chorionic gonadotropin 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 D_1R (C) or LHR (D) significantly activates the JNK activity compared with the basal (Bonferroni paired t test, $P < 0.05$). E, D_1R-induced CREB phosphorylation was inhibited by pretreatment with Rp-cAMPs.

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 D$_1$R-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 μM) effectively suppressed the cAMP/PKA-mediated phosphorylation of CREB upon stimulation with dopamine (Fig. 1E).

Signaling Intermediates of D$_1$R-Mediated JNK Activation. Activation of JNK in response to G$_i$- and G$_q$-coupled receptors seems to depend on G$\beta\gamma$ subunits (Chan and Wong, 2004a,b). 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 D$_1$R, 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 G$\beta\gamma$ subunits are better activators than the α-subunit of G$_s$ (Gα_s) in terms of JNK

activation (Coso et al., 1996). To reveal the identities of G$\beta\gamma$-regulated intermediates for the D$_1$R-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 D$_1$R-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 D$_1$R 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 G$_s$-coupled LHR
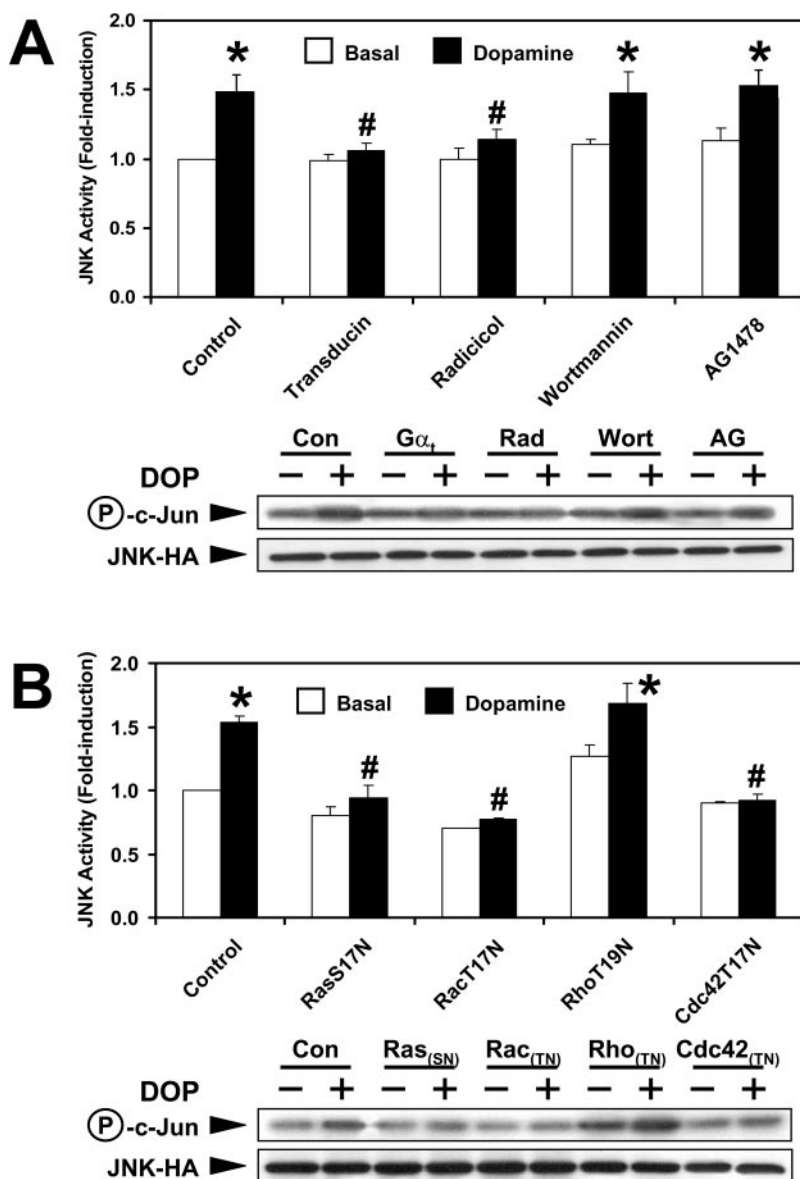

Fig. 2. G$\beta\gamma$ subunits, Src-like kinases, and small GTPases serve as important intermediates for D$_1$R-mediated JNK activation. COS-7 cells were transfected with the cDNAs of JNK-HA and the G$_s$-coupled D$_1$R (A and B). The cells were either coexpressing transducin (Gα_t) for G$\beta\gamma$ 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 G$\beta\gamma$ 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 $G\beta\gamma$, 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). To investigate the integrated JNK activities upon GPCR coactivation, we cotransfected 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 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 ($G\beta\gamma \rightarrow$ Src-like kinase $\rightarrow$ small GTPase $\rightarrow$ 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_i-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_i-, and G_q-coupled receptors use a common $G\beta\gamma$-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_i-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_q signal
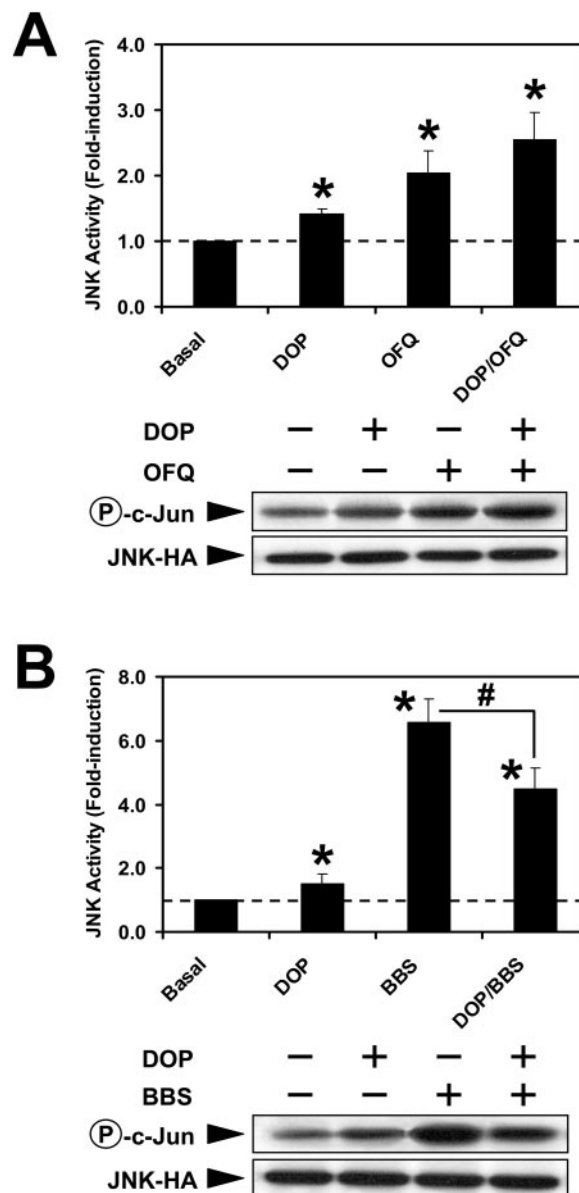
Fig. 3. Differential regulatory effects of G_s-coupled D_1R on the JNK activation triggered by G_i-coupled ORL_1R and G_q-coupled GRPR. COS-7 cells coexpressing JNK-HA and D_1R with ORL_1R (A) or GRPR (B) were stimulated with their corresponding agonists [10 μM dopamine (DOP) for D_1R, 100 nM nociceptin (OFQ) for ORL_1R, and 100 nM bombesin (BBS) for GRPR] separately or simultaneously. The JNK activities were determined 30 min after individual or combinatory treatment of these agonists as indicated. Values shown represent the mean ± S.E. from four separate experiments. ★, individual or combinatory treatment significantly increased the JNK activities compared with the basal. #, costimulation of D_1R significantly inhibited the GRPR-induced JNK activation (Bonferroni paired t test, $P < 0.05$).

from GRPR synergizes with the G_s-mediated adenylyl cyclase activation by D_1R. To investigate the mechanism that 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 $G\beta\gamma$-dependence of adenylyl cyclase activation. For cells cotransfected with transducin, removal of $G\beta\gamma$ subunits (released from G_s and G_q) was incapable of eliminating the synergistic response upon costimulation 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. 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, whereas their coapplication generated synergistic adenylyl cyclase activation (Fig. 4F). These results showed that the G_q-coupled GRPR may use a Ca^{2+} signal to potentiate the adenylyl cyclase activity induced by G_s-coupled D_1R.

Costimulation 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 use both $G\beta\gamma$- as well as PLC-dependent pathways to regulate the JNK activity (Chan and Wong, 2004b). Thus, we examined whether signal integration might have occurred along the G_q/PLC pathway. In COS-7 cells coexpressing G_q-coupled GRPR and G_s-coupled D_1R, treatment with bombesin stimulated IP_3 formation, whereas activation of D_1R by dopamine neither stimulated PLC nor potentiated the GRPR-triggered response (Fig. 5A). Because 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 coexpressing G_s-coupled D_1R and G_i-coupled ORL_1R to determine whether cooperative 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).
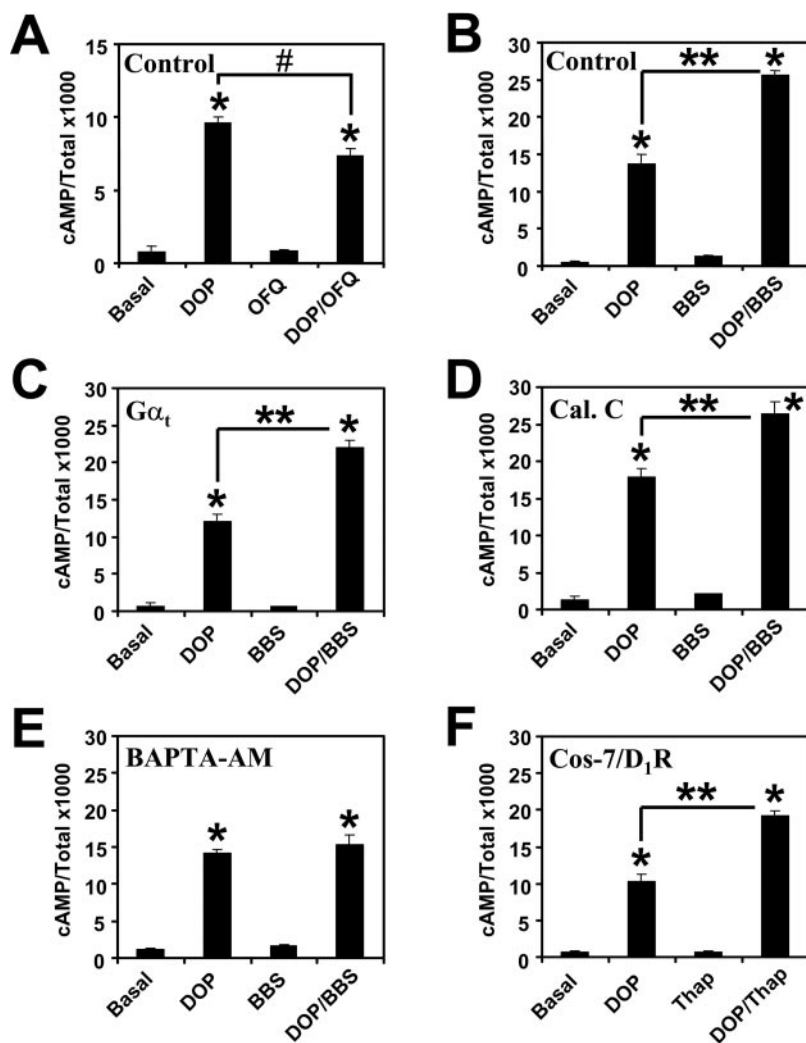

Fig. 4. Modulation of D_1R-mediated adenylyl cyclase activation by G_i-coupled ORL_1R and G_q-coupled GRPR. COS-7 cells coexpressing D_1R with either ORL_1R (A) or GRPR in the absence (B, D, and E) or presence (C) of transducin ($G\alpha_t$) were labeled with [³H]adenine (A–E). These cells were then pretreated with (D and E) or without (A–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, were determined 30 min after individual or costimulation with specific agonists and thapsigargin (Thap, 5 μM) as indicated. Values shown represent the mean ± 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 compared with the basal (A). ∗, treatment with dopamine in the absence or presence of bombesin (B–E) or thapsigargin (F) significantly enhanced the cAMP level compared with the corresponding basal. ∗∗, cotreatment with bombesin (B–D) or thapsigargin (F) significantly increased the D_1R-induced cAMP elevation. #, costimulation of ORL_1R significantly inhibited the D_1R-induced cAMP elevation (Bonferroni paired t test, $P < 0.05$).

cAMP Serves as a Suppressor of G_q-Mediated JNK Activation in COS-7 Cells. The preceding experiments demonstrated that costimulation of D_1R 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 G_q-mediated JNK activation, COS-7 cells were transfected with D_1R 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 G_i-coupled ORL_1R 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 toward Sp-cAMPs (Fig. 7). In addition to the $G\beta\gamma$-dependent pathway, G_q-coupled receptors also require a Ca^{2+} component to stimulate the JNK cascade (Chan and Wong, 2004b). It is possible that cAMP exerts its inhibitory effect on the Ca^{2+}-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 Ca^{2+}-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 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 Ca^{2+} signaling component of the G_q-mediated JNK activity.

Disruption of Ca^{2+} Signaling by BAPTA-AM Suppressed the D_1R/GRPR-Mediated JNK Activity to the D_1R/ORL_1R-Induced Level. As discussed earlier, G_s-, G_i-, and G_q-coupled receptors use the $G\beta\gamma$-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, costimulation 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 subfamilies. To examine this
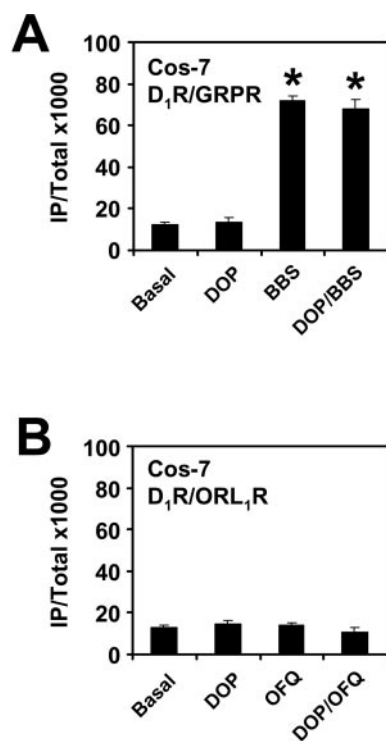

Fig. 5. Costimulation of G_s-coupled D_1R with either G_q-coupled GRPR or G_i-coupled ORL_1R had no effect on PLC. COS-7 cells coexpressing D_1R with either GRPR (A) or ORL_1R (B) were labeled with [myo-^{3}H]inositol for IP_3 assay. The levels of IP_3 formation that reflect the activities of PLC were 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 IP_3 levels compared with the corresponding basal.
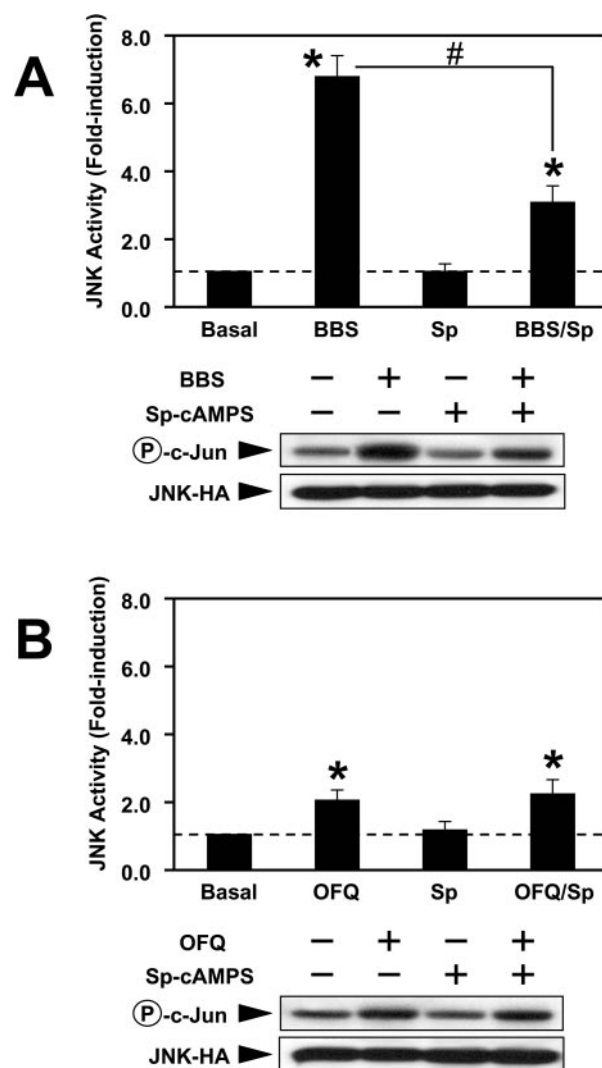

Fig. 6. Sp-cAMPs selectively suppressed the JNK activation mediated by GRPR rather than ORL_1R. COS-7 cells coexpressing JNK-HA and D_1R with either GRPR (A) or ORL_1R (B) were treated with Sp-cAMPs (Sp, 100 μM) and the indicated agonists separately or simultaneously. The JNK activities were determined 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 G$_q$-coupled GRPR and G$_s$-coupled D$_1$R were pretreated with BAPTA-AM to deplete intracellular Ca^{2+}, followed by costimulation with bombesin and dopamine. COS-7 cells coexpressing G$_i$-coupled ORL$_1$R and G$_s$-coupled D$_1$R 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/D$_1$R-induced JNK activity to a level similar to that of the ORL$_1$R/D$_1$R-mediated response (Fig. 8). This finding further demonstrates the importance of Ca^{2+} mobilization for the GRPR/D$_1$R-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 use 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 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 G$_s$-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 corre-

sponding 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.

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
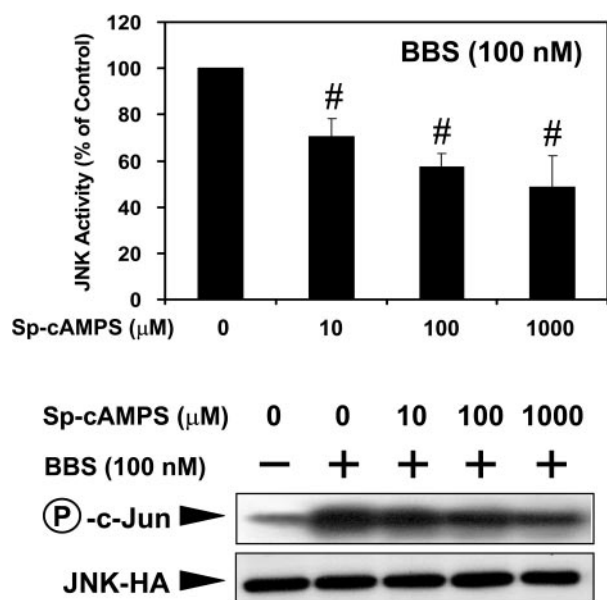
Fig. 8. Disruption of Ca^{2+} signaling by BAPTA-AM suppressed the D$_1$R/GRPR-mediated JNK activity to the D$_1$R/ORL$_1$R-induced level. COS-7 cells coexpressing JNK-HA and D$_1$R with either GRPR or ORL$_1$R were pretreated in the absence (control) or presence of BAPTA-AM (10 μM, 30 min) followed 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 co-stimulation with bombesin and dopamine (Bonferroni paired t test, $P <$ 0.05).

Fig. 7. Sp-cAMPs suppressed the bombesin-induced JNK activation in a dose-dependent manner. COS-7 cells coexpressing JNK-HA, D$_1$R, 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 activation in the absence of Sp-cAMPs defined as 100%. #, cotreatment with Sp-cAMPs significantly inhibited the bombesin-induced JNK activation (Bonferroni paired t test, $P < 0.05$).
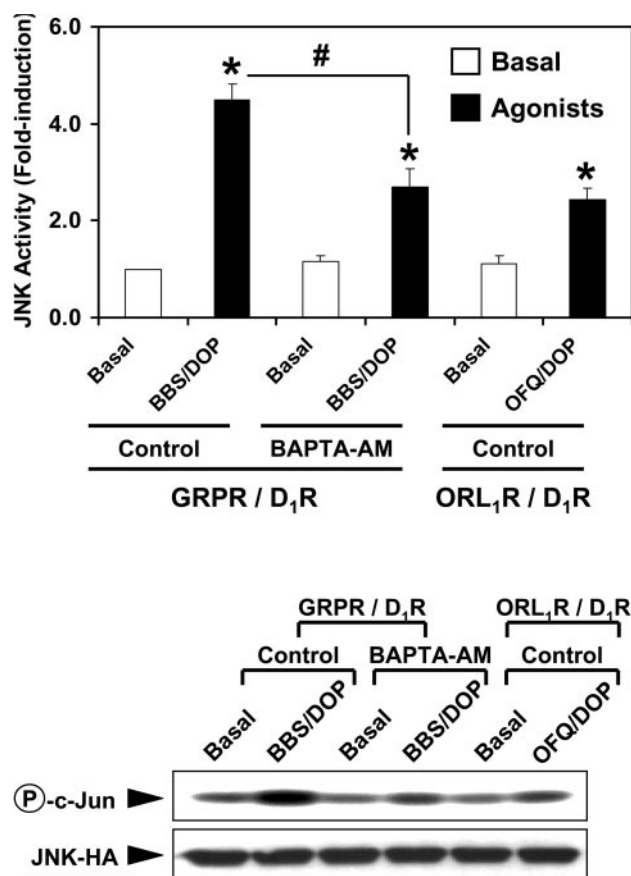
a Rac-dependent manner (Kiyono et al., 2000). In COS-7 cells, both G_i- and G_q-coupled receptors trigger JNK activation via a $G\beta\gamma$/Src-dependent mechanism, which can be suppressed by dominant-negative mutants of both Sos (i.e., Son of Sevenless) and small GTPases (Chan and Wong, 2004b; Kam 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-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_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), 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_s-coupled receptors are required 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 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_3 levels, as well as for JNK activities.

Costimulation of G_s-coupled D_1R and G_i-coupled ORL_1R resulted in an elevated cAMP level which was lower than that of the D_1R response, but no significant enhancement of IP_3 level could be observed (Fig. 5). As illustrated in the present report, cAMP is not as effective as $G\beta\gamma$ subunits for the triggering of JNK activation in COS-7 cells. Hence, the $G\beta\gamma$ subunits released from G_s and G_i may cooperate with each other, using a $G\beta\gamma$/Src-dependent pathway to mediate the JNK activation in a roughly additive manner. The reason for higher JNK-activating capability associated with G_i compared with G_s is currently unknown, but it should be borne in mind that the $G\alpha$ subunits of different G protein members may preferably associate with specific isoforms of $G\beta\gamma$ 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 $G\beta\gamma$ 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 $G\beta\gamma$ subunits to the pathway (Fig. 9A).

The G_s signal neither potentiates nor inhibits the G_q-mediated IP_3 formation in COS-7 cells. In contrast, G_s-induced cAMP elevation is synergistically potentiated by the Ca^{2+} signal from G_q, indicating that the Ca^{2+}-responsive adenylyl 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_s-induced cAMP signaling is associated with an inhibitory function on the Ca^{2+}-dependent JNK pathway. Such an effect may be magnified in the presence of Ca^{2+}-mediated synergistic cAMP formation, which in turn diminishes the cooperative effect between Ca^{2+} and $G\beta\gamma$ of G_q (Chan and Wong, 2004b), resulting in a decreased JNK activation compared with the normal G_q-mediated response
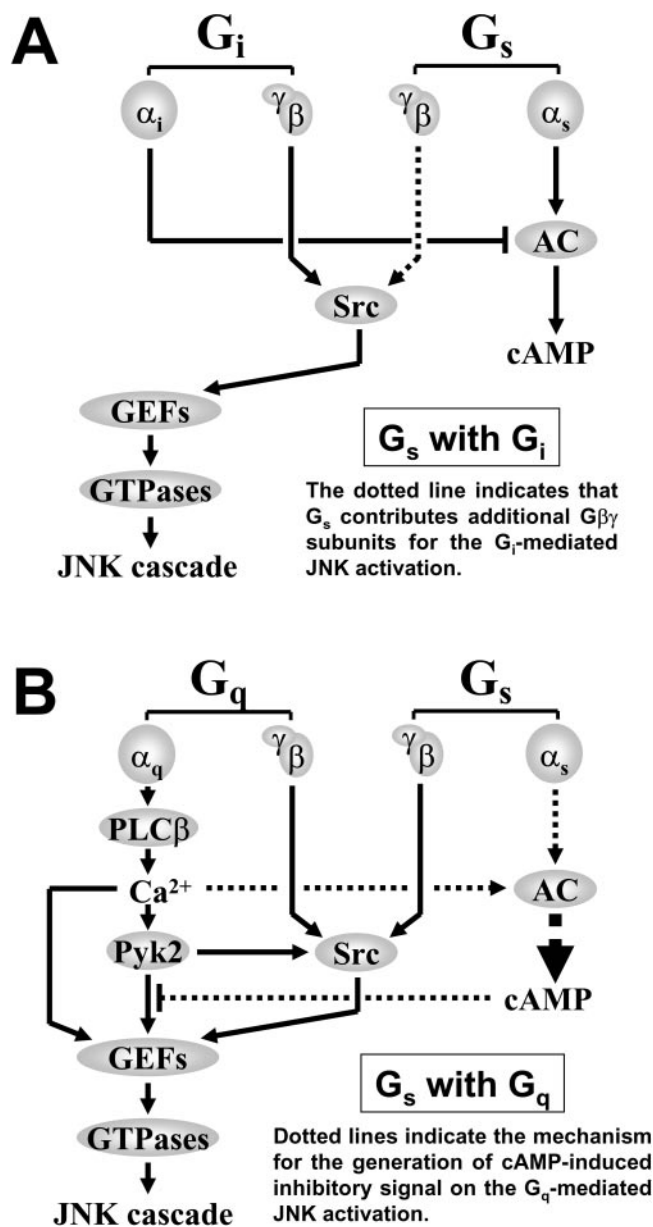

Fig. 9. Schematic diagrams for the regulatory effects of G_s on the G_i- and G_q-mediated JNK activation. A, $G\beta\gamma$ subunits from G_s and G_i converge their signals at Src-like tyrosine kinases and subsequently trigger the activation of JNK cascade, whereas cAMP has no inhibitory effect on the 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 $G\alpha_s$-mediated cAMP elevation is synergistically potentiated in the presence of Ca^{2+}-responsive adenylyl cyclase isoforms. The $G\beta\gamma$/Src and Ca^{2+} signals cooperate with each other to regulate the JNK activation (Chan and Wong, 2004b), whereas cAMP suppresses the induced kinase activity by interfering the Ca^{2+}-mediated pathway.

(Fig. 9B). This idea is supported by our previous finding that activation of vasopressin V_2 receptor (with dual coupling specificities toward 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 toward 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 caused by 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 regarding the possible occurrence of receptor dimerization, 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 G_q/Ca^{2+}-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., $G_q > G_i > G_s$), 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. Because 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 coactivation of G_s-coupled receptors, resulting in a kinase activity lower than the 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 costimulation of G_s-coupled receptor. Taking all of 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 and 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 that determine cell fate such as proliferation, differentiation, or apoptosis (Kobayashi and Tsukamoto, 2001). Further investigation should be focused on suitable endogenous cellular systems to define the possible biological consequence of this integrated JNK activity.

References

Albert PR and Robillard L (2002) G protein specificity: traffic direction required. *Cell Signal* **14**:407–418.

Balmforth AJ, Ball SG, Freshney RI, Graham DI, McNamee HB, and Vaughan PF (1986) D_1 dopaminergic and β-adrenergic stimulation of adenylate cyclase in a clone derived from the human astrocytoma cell line G-CCM. *J Neurochem* **47**:715–719.

Chan AS, Lai FP, Lo RK, Voyno-Yasenetskaya TA, Stanbridge EJ, and Wong YH (2002) Melatonin MT_1 and MT_2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and -insensitive G proteins. *Cell Signal* **14**:249–257.

Chan AS and Wong YH (2000) Regulation of c-Jun N-terminal kinase by the ORL_1 receptor through multiple G proteins. *J Pharmacol Exp Ther* **295**:1094–1100.

Chan AS and Wong YH (2004a) Epidermal growth factor differentially augments G_i-mediated stimulation of c-Jun N-terminal kinase activity. *Br J Pharmacol* **142**:635–646.

Chan AS and Wong YH (2004b) $G\beta\gamma$ signaling and Ca^{2+} mobilization co-operate synergistically in a Sos and Rac-dependent manner in the activation of JNK by G_q-coupled receptors. *Cell Signal* **16**:823–836.

Chan JSC, Lee JWM, Ho MKC, and Wong YH (2000) Preactivation permits subsequent stimulation of phospholipase C by G_i-coupled receptors. *Mol Pharmacol* **57**:700–708.

Coso OA, Teramoto H, Simonds WF, and Gutkind JS (1996) Signaling from G protein-coupled receptors to c-Jun kinase involves $\beta\gamma$ subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J Biol Chem* **271**:3963–3966.

Das B, Shu X, Day GJ, Han J, Krishna UM, Falck JR, and Broek D (2000) Control of intramolecular interactions between the pleckstrin homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. *J Biol Chem* **275**:15074–15081.

Dunn C, Wiltshire C, MacLaren A, and Gillespie DA (2002) Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cell Signal* **14**:585–593.

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, and Bos JL (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* **4**:901–906.

Feoktistov I, Goldstein AE, and Biaggioni I (1999) Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A_{2B} receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol* **55**:726–734.

Gerhardt CC, Gros J, Strosberg AD, and Issad T (1999) Stimulation of the extracellular signal-regulated kinase 1/2 pathway by human β_3 adrenergic receptor: new pharmacological profile and mechanism of activation. *Mol Pharmacol* **55**:255–262.

Harada T, Morooka T, Ogawa S, and Nishida E (2001) ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat Cell Biol* **3**:453–459.

Hochbaum D, Tanos T, Ribeiro-Neto F, Altschuler D, and Coso OA (2003) Activation of JNK by Epac is independent of its activity as a Rap guanine nucleotide exchanger. *J Biol Chem* **278**:33738–33746.

Hur EM and Kim KT (2002) G protein-coupled receptor signaling and cross-talk achieving rapidity and specificity. *Cell Signal* **14**:397–405.

Kam AY, Chan AS, and Wong YH (2003) Rac and Cdc42-dependent regulation of c-Jun N-terminal kinases by the δ-opioid receptor. *J Neurochem* **84**:503–513.

Kam AY, Chan AS, and Wong YH (2004) κ-opioid receptor signals through Src and focal adhesion kinase to stimulate c-Jun N-terminal kinases in transfected COS-7 cells and human monocytic THP-1 cells. *J Pharmacol Exp Ther* **310**:301–310.

Kanno Y, Tokuda H, Nakajima K, Ishisaki A, Shibata T, Numata O, and Kozawa O (2004) Involvement of SAPK/JNK in prostaglandin E_1-induced VEGF synthesis in osteoblast-like cells. *Mol Cell Endocrinol* **220**:89–95.

Kiyono M, Kaziro Y, and Satoh T (2000) Induction of rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm) following phosphorylation by the non-receptor tyrosine kinase Src. *J Biol Chem* **275**:5441–5446.

Kobayashi K and Tsukamoto I (2001) Prolonged Jun N-terminal kinase (JNK) activation and the upregulation of p53 and p21(WAF1/CIP1) preceded apoptosis in hepatocytes after partial hepatectomy and cisplatin. *Biochim Biophys Acta* **1537**:79–88.

Laroche-Joubert N, Marsy S, Michelet S, Imbert-Teboul M, and Doucet A (2002) Protein kinase A-independent activation of ERK and H,K-ATPase by cAMP in native kidney cells: role of Epac I. *J Biol Chem* **277**:18598–18604.

Li X, Yu H, Graves LM, and Earp HS (1997) Protein kinase C and protein kinase A inhibit calcium-dependent but not stress-dependent c-Jun N-terminal kinase activation in rat liver epithelial cells. *J Biol Chem* **272**:14996–15002.

Lopez-Ilasaca M, Gutkind JS, and Wetzker R (1998) Phosphoinositide 3-kinase γ is a mediator of $G_{\beta\gamma}$-dependent Jun kinase activation. *J Biol Chem* **273**:2505–2508.

Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, and Lefkowitz RJ (1997) Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J Biol Chem* **272:**4637–4644.

McCawley LJ, Li S, Benavidez M, Halbleib J, Wattenberg EV, and Hudson LG (2000) Elevation of intracellular cAMP inhibits growth factor-mediated matrix metallo-proteinase-9 induction and keratinocyte migration. *Mol Pharmacol* **58:**145–151.

Mochizuki N, Ohba Y, Kobayashi S, Otsuka N, Graybiel AM, Tanaka S, and Matsuda M (2000) Crk activation of JNK via C3G and R-Ras. *J Biol Chem* **275:**12667–12671.

Murga C, Fukuhara S, and Gutkind JS (2000) A novel role for phosphatidylinositol 3-kinase β in signaling from G protein-coupled receptors to Akt. *J Biol Chem* **275:**12069–12073.

Pfleger KD and Eidne KA (2005) Monitoring the formation of dynamic G-protein coupled receptor-protein complexes in living cells. *Biochem J* **385 (Pt 3):**625–637.

Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, and Lefkowitz RJ (2001) Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* **276:**23155–23160.

Reinehr R, Becker S, Hongen A, and Haussinger D (2004) The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor receptor and CD95. *J Biol Chem* **279:**23977–23987.

Rossant CJ, Pinnock RD, Hughes J, Hall MD, and McNulty S (1999) Corticotropin-releasing factor type 1 and type 2α receptors regulate phosphorylation of calcium/ cyclic adenosine 3′,5′-monophosphate response element-binding protein and activation of p42/p44 mitogen-activated protein kinase. *Endocrinology* **140:**1525–1536.

Selbie LA and Hill SJ (1998) G protein-coupled receptor cross-talk: the fine-tuning of multiple receptor signaling pathways. *Trends Pharmacol Sci* **19:**87–93.

Wayman GA, Impey S, Wu Z, Kindsvogel W, Prichard L, and Storm DR (1994) Synergistic activation of the type I adenylyl cyclase by Ca^{2+} and G_s-coupled receptors in vivo. *J Biol Chem* **269:**25400–25405.

Widmann C, Gibson S, Jarpe MB, and Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* **79:**143–180.

Yamauchi J, Hirasawa A, Miyamoto Y, Itoh H, and Tsujimoto G (2001) $β_2$-adrenergic receptor/cycle adenosine monophosphate (cAMP) leads to JNK activation through Rho family small GTPases. *Biochem Biophys Res Commun* **284:**1199–1203.

Zhen X, Uryu K, Wang HY, and Friedman E (1998) D_1 dopamine receptor agonists mediate activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase by a protein kinase A-dependent mechanism in SK-N-MC human neuro-blastoma cells. *Mol Pharmacol* **54:**453–458.

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