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Cell Contact-dependent Functional Selectivity of β_2-Adrenergic Receptor Ligands in Stimulating cAMP Accumulation and Extracellular Signal-regulated Kinase Phosphorylation*

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Background: β_2-Adrenegic receptor (β_2-AR) mediates cAMP accumulation and ERK phosphorylation via different transducers.
Results: Some β_2-AR ligands selectively activate cAMP and ERK responses in HEK-293 cells. Selectivity is cell contact-dependent.
Conclusion: β_2-AR stimulates ERK phosphorylation by cAMP-dependent and G_i/G_o-mediated pathways. Cell contact inhibits the cAMP-dependent pathway.
Significance: Cell adherence/contact is an important modulatory factor in biased signaling by GPCR ligands.

Activation of β_2-adrenegic receptor (β_2-AR) leads to an increase in intracellular cAMP and activation of ERK. These two signals are activated by the interaction of the receptor with different transducer partners. We showed that the intrinsic activities of β_2-AR ligands for stimulating cAMP production and ERK phosphorylation responses in HEK-293 cells were not correlated. The lack of correlation resulted mainly from the discrepancy between the intrinsic activities of two groups of ligands for these two responses: The first group consisted of clenbuterol, cimaterol, procaterol, and terbutaline which acted as full agonists for cAMP production but displayed very weak effect on ERK phosphorylation. The second group comprised adrenaline and noradrenaline which displayed higher intrinsic activity for the ERK phosphorylation than for the cAMP response. Thus, both groups behaved as functionally selective ligands. The functional selectivity of the first group was observable only in adherent cells when confluence was approximately 100%. When cell-cell contact was minimized either by decreasing the density of the adherent cells or by bringing the cells into suspension, the first group of ligands gained the ability to stimulate ERK phosphorylation without a change in their effect on cAMP production. In contrast, selectivity of the second group was independent of the adherence state of the cells. Our results show that the inherent "bias" of ligands in coupling a G protein-coupled receptor to different transducers may not always be revealed as functional selectivity when there is a "cross-talk" between the signaling pathways activated by the same receptor.

The signal transduction mechanism of G protein-coupled receptor (GPCR)[3] systems has long been explained by a linear signal flow scheme in which activation of one type of receptor activates one type of G protein, which, in turn, usually activates one effector (1, 2). Accordingly, receptors have been classified as G_s-, G_i-, or G_q-coupled depending on the type of G protein they activate. Receptor ligands, on the other hand, have been categorized as agonists, antagonists, or inverse agonists according to their ability to activate (or inactivate) the signal pathway associated with their target receptor. However, it gradually became evident in the past 15 years that one type of receptor can in fact activate branching pathways by interacting with more than one signaling partner (3), including more than one type of G protein (see Ref. 4 and references therein). It is now a widely appreciated phenomenon that receptor ligands can selectively activate these signaling pathways, provided that the branching occurs at the level of receptor-transducer interaction (for a review, see Ref. 5). Thus, a ligand that is an agonist in terms of a signaling pathway may behave as an antagonist for another pathway associated with the same receptor. Identification of this phenomenon, named as functional selectivity (6), is an important development in pharmacology because it provides a new conceptual framework for understanding both the drug effects (and side effects) and the molecular mechanisms of receptor activation.

β_2-Adrenergic receptor (β_2-AR) is a prototypical G_s-coupled receptor that mediates intracellular cAMP increase by activating G_s (7). Hence, efficacy assignment to β_2-AR ligands has

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; β_2-AR, β_2-adrenergic receptor; CREB, cAMP-responsive element-binding protein; db-cAMP, dibutyryl cyclic AMP; MTT, 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide; myr-PKI, myristoylated (14–22)-fragment of cAMP-dependent protein kinase inhibitor peptide; pERK, phosphorylated ERK; PTX, pertussis toxin; tERK, total ERK.

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been based on the ability of the ligands to activate adenylyl cyclase. Relatively more recently, it has been shown that β_2-AR could also mediate extracellular signal-regulated kinase (ERK) activation (8–10). Although the signaling pathway(s) that couple β_2-AR to ERK activation have not been fully understood, strong experimental evidence suggests that the signal is initiated upon the interaction of the activated receptor, in addition to G_s, with G_i (10, 11), Src (12), and/or arrestin (11) proteins. As such, β_2-AR-induced cAMP and ERK responses constitute an excellent example for the signal branching at the level of receptor-transducer interaction. Available data concerning the functional selectivity of β-AR ligands suggest that some ligands may selectively activate these two responses, as they behave as antagonists or inverse agonists in terms of cAMP response but as agonists in terms of ERK activation (13, 14). However, a detailed screening of β_2-AR ligands for their efficacy in ERK phosphorylation response has not yet been reported.

The above mechanisms of β_2-AR-initiated ERK activation set aside, intracellular cAMP itself is known to induce or inhibit ERK phosphorylation, depending on the cell type (15–17). To explain such opposite effects, Vossler *et al.* proposed a model wherein cAMP causes a Rap 1-mediated ERK phosphorylation in cells that express 95-kDa isoform of B-Raf, but inhibits Ras-mediated ERK phosphorylation in cells that lack the B-Raf isoform (16). However, heterologous expression of B-Raf did not always restore the cAMP-induced ERK activation in cells lacking the relevant protein (17). Therefore, mechanisms underlying the cell type-dependence of cAMP-mediated ERK phosphorylation remain unclear. Irrespective of the mechanism(s) involved, in those cells where cAMP is a sufficient signal for ERK phosphorylation, all β_2-AR ligands that increase intracellular cAMP are expected to cause ERK phosphorylation as well. Surprisingly, however, we observed that some β_2-AR ligands that are strong agonists for intracellular cAMP accumulation were unable to induce ERK phosphorylation in HEK-293 cells, a cell line in which cAMP analogs or forskolin, a direct stimulator of adenylyl cyclase, have been shown to cause phosphorylation of ERK (18, 19). We also observed that ligands displayed this "functional selectivity" conditionally, depending on the adherence state of the cells. Here, we present a detailed analysis of the β_2-AR ligand efficacies in terms of cAMP accumulation and ERK phosphorylation responses in different adherence conditions and propose a potential mechanism for the conditional nature of the functional selectivity that ligands display.

EXPERIMENTAL PROCEDURES

Materials

Cell culture media and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). Receptor ligands were from either Tocris (Ellisville, MO) or Sigma. Forskolin, dibutyryl-cAMP (db-cAMP), isobutylmethylxanthine, MTT, myristoylated protein kinase A inhibitor peptide (myr-PKI) and pertussis toxin (PTX) were from Sigma. Complete Mini protease inhibitors were from Roche Diagnostics. ^{125}I was purchased from Amersham Biosciences. Primary and HRP-conjugated secondary antibodies, enhanced chemiluminescence substrate, siRNAs targeting the α and β catalytic subunits of PKA (sc-36240 and sc-39158, respectively), and control siRNA (sc-37007) were from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000™ was from Invitrogen.

Cell Culture

HEK-293 cells that stably over express β_2-AR were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) FBS, at 37 °C in a humidified atmosphere of 5% CO_2. The expression level of β_2-AR in this stable clone is 30 pmol/mg membrane protein as assessed by ^{125}I-cyanopindolol binding in membrane preparations.

ERK Phosphorylation Assay

Adherent Cells—Unless indicated otherwise, assay was conducted with confluent cells in uncoated 6-well plates (Greiner Bio-One, Frickenhausen, Germany). On the day of the experiment, cells were washed twice with serum-free DMEM and incubated in the same medium at least for 2 h. The assay was initiated by adding the indicated ligands or stimulants at 37 °C. At the end of the incubation, medium was removed, cells were scraped into 100 μl of ice-cold lysis buffer (1% Nonidet P-40, 0.2 mM $Na(VO_4)$, and protease inhibitor mix in PBS) and lysed on ice for 15 min. Lysates were cleared by centrifugation at 10,000 $\times$ *g* for 5 min.

Suspended Cells—On the day of experiment, cells grown to confluence in culture flasks were washed twice with serum-free DMEM and incubated in the same medium at least for 2 h. Cells were then detached by EDTA (1 mM, in PBS), pelleted, resuspended in serum-free DMEM at a density of 3×10^6 cells/ml, and distributed to 96-well ultralow binding plates (Corning) in 100 μl. After a 30–40-min resting period, the assay was initiated by adding the receptor ligands or stimulants (50 μl) and stopped by adding 4× ice-cold lysis buffer (50 μl). Lysates were cleared by centrifugation at 10,000 $\times$ *g* for 5 min.

The effect of PTX on ERK phosphorylation responses were assessed in cells that were incubated overnight with PTX (100 ng/ml) in serum-free medium. In some experiments, cells were preincubated with H89 (10 μM) or myr-PKI (30 μM) for 20 min in serum-free medium at 37 °C.

For both the adherent and the suspended cells, a time-dependent ERK phosphorylation experiment typically involved four to eight ligands one of which was always isoproterenol. Peak ERK phosphorylation responses induced in 3 min were assessed in parallel for all 16 ligands, forskolin and db-cAMP at least in three independent experiments run in duplicate. Isoproterenol was used in all experiments as the control ligand to assess the daily performance of the cells in terms of ERK phosphorylation response. Responses induced by ligands (or forskolin or db-cAMP) were normalized with that induced by isoproterenol in the parallel experiment to minimize the interexperimental variation.

Immunoblotting

Samples of equal volume from the cleared lysates were separated on 10% SDS-PAGE, transferred to PVDF membranes, and immunoblotted using first an antibody specific for the phos-

phorylated forms of ERK1/2 (pERK). After stripping, membranes were re-probed by an ERK2-specific antibody to assess the total ERK (tERK) amounts in the samples. Protein bands were detected using appropriate HRP-conjugated secondary antibodies and enhanced chemiluminescence as described by the manufacturer. A CCD camera system and an image analysis software (AIDA; Raytest, Straubenhardt, Germany) were used for the densitometric analysis of the protein bands. Band densities in pERK blots were corrected with the tERK densities in the corresponding samples. When multiple gels were used to run the samples of a single experiment, a reference sample was run on each gel, analyzed for pERK and ERK densities, and used as a standard to minimize the potential intrablot variation.

Determination of cAMP Accumulation in Intact Cells

Adherent Cells—Cells were seeded in 96-well plates (~4×10^4 cells/well) the day before the experiment. On the day of experiment, cells were washed once with serum-free DMEM and incubated in the same medium for at least 2 h.

Suspended Cells—On the day of experiment, cells grown to confluence in culture flasks were washed once with serum-free DMEM and incubated in the same medium at least for 2 h. Cells were then detached by EDTA (1 mM, in PBS), pelleted, resuspended in serum-free DMEM at a density of 4×10^5 cells/ml, and distributed to 96-well plates in 100 μl. Both the adherent and the suspended cells were incubated with the receptor ligands for 3 min before the assay. The assay was started by the addition of isobutylmethylxanthine (1 mM) and terminated after 5 min by the addition of HCl at a final concentration of 0.1 N. The amount of cAMP formed was measured by radioimmunoassay as described before (20). Cyclic AMP accumulations were corrected with the cell viability determined by MTT assay in cells seeded (adherent cells) or suspended and preincubated in exactly the same conditions with the cells used in the parallel cAMP assay.

siRNA Transfection

Transfections were performed according to the method described by Liu *et al.* (21). Cells at a confluence of 70% were transfected in 6-well plates with siRNAs targeting α and β catalytic subunits of PKA (100 pmol/well, each) and nontargeting siRNA (200 pmol/well), using Lipofectamine 2000, according to the recommendations of the manufacturer. After a 6-h incubation, transfection medium was replaced with DMEM containing 5% FCS. Transfection was repeated the following day, using the same amounts of siRNAs. One well of cells was split into 8 wells of 24-well plates at the 24th h and used for the ERK phosphorylation assay on the 48th h of the second transfection. Expression levels of PKA subunits were checked in transfected cell lysates by immunoblotting, using antibodies that specifically recognize α or β catalytic subunits of PKA.

Bioluminescence Resonance Energy Transfer Assay of β_2-AR-G Protein and β_2-AR-Arrestin Interactions

The intrinsic activities of β_2-AR ligands for promoting receptor-G protein or receptor-arrestin coupling were measured by bioluminescence resonance energy transfer. G protein interaction was determined in membrane preparations, whereas the arrestin interaction was recorded in intact cells, using HEK-293 cells co-expressing the luminescent β_2-AR-Rluc and either RGFP-Gβ_1 or RGFP-β-arrestin2, as described in detail previously (22). To compare the extent of receptor-arrestin interaction between adherent cells and suspensions, cells (300,000 cells/well) were either seeded in 96-well plates 24 h prior to the assay (adherent state) or added as freshly prepared suspension to each well. In both cases, cells were preincubated for 3 min with 5 μM coelenterazine before the addition of various concentrations of the ligands. After 5 min of further incubation, luminescence was counted in a plate luminometer (VICTOR light; PerkinElmer Life Sciences), and the bioluminescence resonance energy transfer ratio was determined as described (22).

Statistical Analysis

We used different variants of Student's *t* statistics whenever appropriate. Pearson's correlation coefficient was used to measure the association between different variables. We considered $p < 0.05$ statistically significant.

RESULTS

We screened the intrinsic activities of 18 β_2-AR ligands in terms of intracellular cAMP accumulation and ERK phosphorylation responses in a HEK-293 cell clone that stably expresses β_2-AR (HEK-β cells). In terms of adenylyl cyclase activation, the set of the β_2-AR ligands used in this study contained (i) inverse agonists ICI-118,551 and timolol; (ii) neutral ligands sotalol, ICI-89406, and propranolol; (iii) partial agonists cyanopindolol, pronethanol, alprenolol, pindolol, dobutamine, noradrenaline, and dopamine; and (iv) apparent full agonists adrenaline, clenbuterol, terbutaline, cimaterol, procaterol, and isoproterenol.

In HEK-β cells, all ligands that were able to induce ERK phosphorylation produced a typical transient response with a peak at the 3rd min (Fig. 1). We assessed ERK phosphorylation signal at the 3rd min as the measure of the ability of the ligand to produce ERK response. We measured ligand-induced cAMP accumulation in 5 min in intact cells and used the amount of cAMP produced as the measure of the ability of the ligand to activate adenylyl cyclase. In all assays, ligands were used at saturating concentrations.

Some β_2-AR Ligands Behave Selectively in Stimulating Adenylyl Cyclase Activation and ERK Phosphorylation

In 70–100% confluent cells, isoproterenol, adrenaline, noradrenaline, and dopamine induced a strong ERK phosphorylation response, whereas procaterol, terbutaline, cimaterol, and clenbuterol, did not or only weakly stimulated ERK phosphorylation (Fig. 1). The ERK responses to the latter four ligands were quite variable among experiments (from none to a small detectable signal). Among the ligands that induced the ERK response, only adrenaline and noradrenaline caused detectable ERK phosphorylation signals in untransfected HEK-293 cells. Both signals were inhibited almost completely by a specific β_2-AR antagonist, ICI-118,551 (Fig. 1*B*), showing that the contribution of endogenously expressed adrenergic receptor subtypes (other than β_2-AR) to the ERK responses in HEK-β cells was insignificant in the present experimental conditions. Thus,

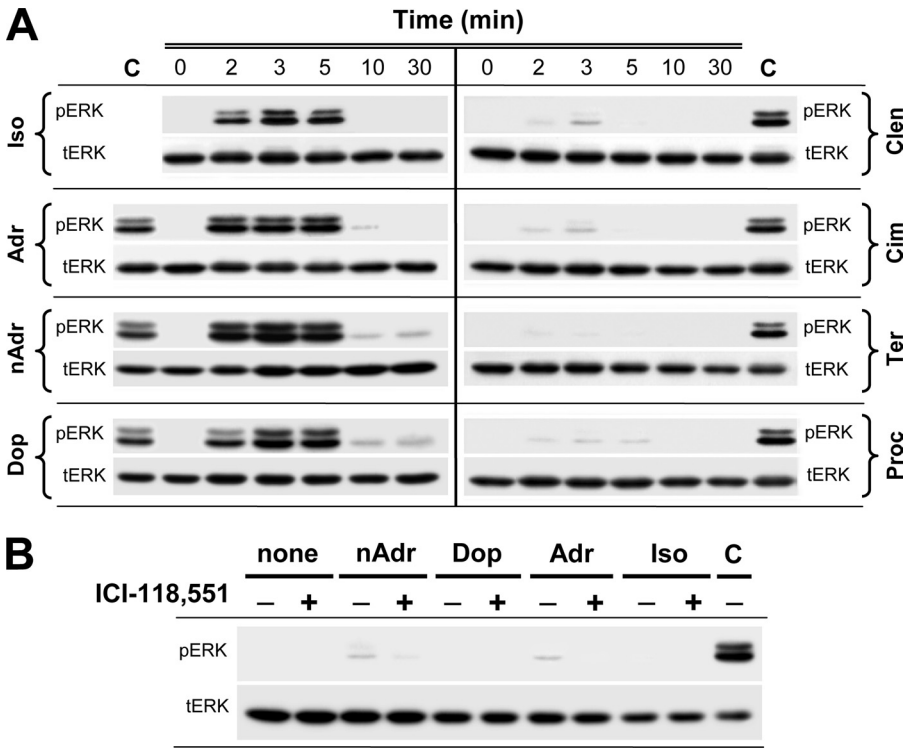


FIGURE 1. **Some β_2-AR ligands that are strong agonists for adenylyl cyclase activation do not stimulate ERK phosphorylation in confluent cells.** *A*, HEK-β cells were grown to confluence and stimulated with isoproterenol (*Iso*, 10^{-5} M), adrenaline (*Adr*, 10^{-5} M), noradrenaline (*nAdr*, 10^{-4} M), dopamine (*Dop*, 10^{-4} M), clenbuterol (*Clen*, 10^{-5} M), cimaterol (*Cim*, 10^{-5} M), procaterol (*Proc*, 10^{-5} M), or terbutaline (*Ter*, 10^{-5} M) for the indicated times. *B*, untransfected HEK-293 cells were grown to confluence and stimulated with the indicated ligands for 3 min in the absence or presence of 10^{-5} M ICI-118,551. Final concentrations of the ligands are the same with the ones used in *A*. After stimulation, cells were lysed, and samples of equal volume from the lysates were analyzed by Western blotting using first an antibody specific to the phosphorylated form of ERK. After stripping, membranes were reprobed by an ERK-specific antibody to determine the total amount of the ERK protein in samples. The HEK-β cell lysate from the 3rd-min isoproterenol stimulation was run in all gels (except the gel that already contains that sample) as a reference and is indicated as *C* in the picture. Shown are the representatives of at least three experiments with similar results.

procaterol, terbutaline, cimaterol, and clenbuterol that are full agonists for increasing cAMP seemed to behave as antagonists in activating ERK, whereas isoproterenol, adrenaline, noradrenaline, and dopamine behaved as agonists for both the cAMP and the ERK phosphorylation responses. Dobutamine, a partial agonist for cAMP production, reproducibly caused a small but significant ERK phosphorylation response, suggesting that it behaved as a partial agonist for both responses (data not shown, but see below). These results showed that the ligand efficacies for cAMP and ERK responses were not correlated and, by definition, indicated that some β_2-AR ligands were functionally selective in terms of adenylyl cyclase activation and ERK phosphorylation.

cAMP Induces ERK Phosphorylation Conditionally in HEK-β Cells

Besides pointing to a functional selectivity, the lack of correlation between ligand-induced cAMP production and ERK phosphorylation also implied that the two responses were independent. In other words, cAMP produced by β_2-AR stimulation did not stimulate ERK phosphorylation directly because some of the ligands tested here were not able to stimulate ERK phosphorylation despite their ability to increase intracellular cAMP to a high level. Apparently, the latter observation is inconsistent with the substantial data in the literature showing that forskolin and cAMP analogs are able to stimulate ERK phosphorylation in the HEK-293 cell line. Indeed, forskolin, as well as a cell-permeable cAMP analog db-cAMP, caused ERK phosphorylation in the present experimental system, suggesting that cAMP was able to induce ERK phosphorylation in our hands as well. This result, at first, seemed to suggest that procaterol, terbutaline, cimaterol, and clenbuterol were activating an additional signal that inhibited the cAMP-mediated ERK phosphorylation. However, in the course of the experiments, we noticed a striking variation in the forskolin-induced response (Fig. 2*A*). We then found out that the variation in forskolin-induced ERK response could be explained almost completely by the variation in cell confluence: the response gradually decreased and eventually ceased as the cell confluence increased and reached 100% (Fig. 2*B*). Confluence had a small effect on isoproterenol-induced ERK phosphorylation as well, but this effect was insignificant compared with the one on the forskolin response. As expected, the effect of confluence on db-cAMP-induced ERK response was parallel to that on forskolin response (Fig. 2*D*). These results showed that cAMP could induce ERK phosphorylation in HEK-β cells conditionally, depending on the confluence state of the culture.

An obvious result of the increased cell confluence is the overall increase in the contact surface between cells, or vice versa. Hence, a possible explanation for the modulatory effect of cell confluence on the cAMP-induced ERK response would be the

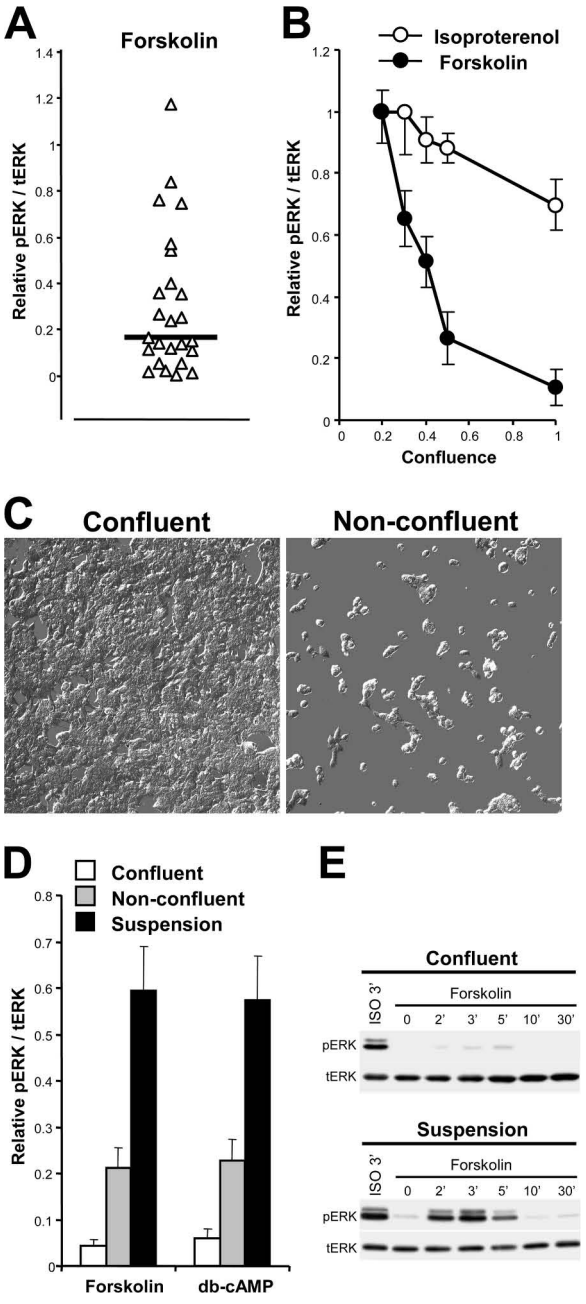


FIGURE 2. **cAMP-induced ERK phosphorylation is confluence-dependent in HEK-β cells.** *A*, parallel obtained isoproterenol- or forskolin-induced ERK phosphorylation responses in 3 min in attached HEK-β cells of varying confluence were gathered together to show the considerable variation in forskolin-induced ERK phosphorylation response. Confluence of the cells on the day of the experiment was not determined systematically and varied between 20 and 100%. Phospho-ERK band densities were corrected with the total ERK band densities in each sample and designated as peak ERK phosphorylation signal for each stimulant. Each data point in the picture represents the peak forskolin-induced ERK phosphorylation relative to the parallel obtained peak isoproterenol-induced ERK phosphorylation in independent experiments. The median is also shown as a *thick line*. *B*, decreasing amounts of HEK-β cells starting from 2.5×10^6 cells/well were seeded to the 6-well culture plates the day before the ERK phosphorylation assay. The *x* axis shows the seeding dilutions which roughly correspond to the confluence on the day of the experiment, *1* representing 100% confluence. Cells were stimulated with isoproterenol or forskolin for 3 min, lysed, and analyzed for phospho-ERK (*pERK*) and total ERK (*tERK*) immunoreactivities in the lysates. Data are the mean ± S.E. (*error bars*) of three independent experiments. *C*, cells were seeded at a density of 1×10^6 (*left*) or 1×10^5 (*right*) cells/well onto the glass coverslips in 6-well culture plates and were visualized on the 48th (*left*) or 24th (*right*) h, using a light microscope with a differential interference contrast attachment. Intercontacting cell groups are visible in low density culture conditions on the *left panel*. *D*, peak ERK phosphorylations in response to forskolin (10^{-5} M) or db-cAMP (10^{-5} M) were measured in 40–50% confluent or in suspended cells. Responses were presented relative to the peak isoproterenol-induced ERK phosphorylation obtained in parallel. Data are the mean ± S.E. of at least six independent determinations for each condition. *E*, adherent confluent or suspended HEK-β cells were stimulated with 10^{-5} M forskolin for indicated times, lysed, and analyzed by Western blotting. HEK-β cell lysates of the 3rd-min isoproterenol stimulation (*ISO 3'*) in each condition were run in gels as reference.

inhibition of cAMP-mediated ERK phosphorylation by cell-cell contact. To characterize ERK response in cells where cell-cell contact is minimized, we measured ERK phosphorylation in suspended cells. The reason for not using adherent cells in these experiments was that groups of 3–10 intercontacting cells, due to the growth characteristics of HEK-293 cells, were visible on the day of experiment even when the cells were plated at a very low density (Fig. 2*C*). Therefore, we grew the cells to confluence, detached them from the culture flask by using EDTA, and then washed and resuspended them in serum-free DMEM. Both the db-cAMP- and the forskolin-induced peak ERK phosphorylation responses were significantly increased in suspended cells compared with the responses in both 100 and 50% confluent cells (Fig. 2*D*). The time course of the forskolin-induced ERK phosphorylation response in suspended cells was similar to that of the isoproterenol-induced one in adherent cells (Fig. 2*E*).

Functional Selectivity of β_2-AR Ligands Is Lost When cAMP Can Induce ERK Phosphorylation

The above results led to the question as to whether clenbuterol, cimaterol, procaterol, and terbutaline would induce ERK phosphorylation in the conditions where the intracellular cAMP increase was sufficient for ERK activation. Thus, we examined the ligand-induced ERK phosphorylation responses in reduced cell-cell contact conditions. In contrast to the situation in confluent cells, all of the cAMP agonists, including clenbuterol, cimaterol, procaterol, and terbutaline, were indeed able to stimulate ERK phosphorylation in both 40–50% confluent (data not shown, but see below) and in suspended cells (Fig. 3). Thus, clenbuterol, cimaterol, procaterol, and terbutaline, the ligands that behaved as antagonists in terms of ERK phosphorylation in confluent cells, converted into agonists for the same response when the cell-cell contact was reduced either by reducing the confluence or by bringing the cells into suspension. These results showed that clenbuterol, cimaterol, procaterol, and terbutaline were able to induce ERK phosphorylation only when the intracellular cAMP increase could induce ERK phosphorylation. They also indicated that, in confluent cells, adrenaline, noradrenaline, dopamine, and isoproterenol used a cAMP-independent pathway to induce ERK phosphorylation.

We then screened 18 β_2-AR ligands for their ability to induce ERK phosphorylation in 100% and 50% confluent and in suspended cells and assessed the ligand-induced ERK phosphorylation at the 3rd min as the measure of ligand intrinsic activity for ERK activation. Intrinsic activities of the ligands in terms of cAMP accumulation response were not affected by the adherence state of the cells. Neither the order nor the absolute mag-

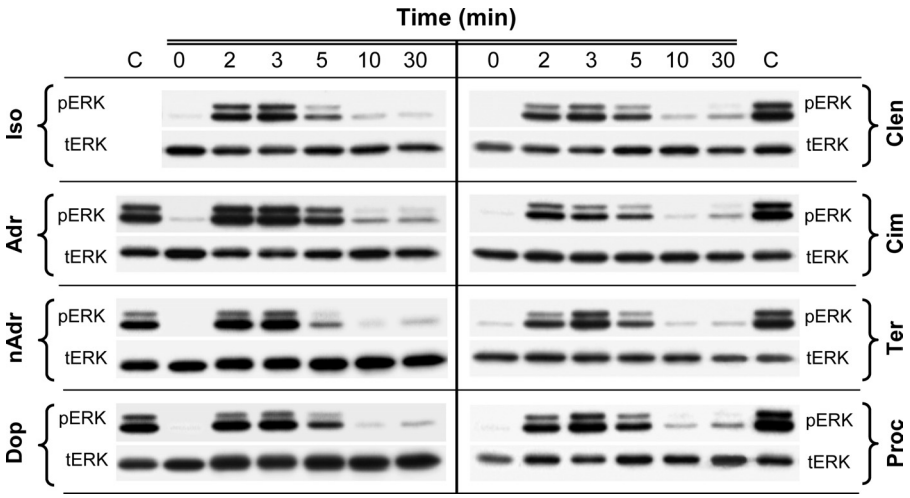


FIGURE 3. **All ligands that stimulate cAMP accumulation also stimulate ERK phosphorylation in suspended cells.** *A*, HEK-β cells grown to confluence were suspended and stimulated with the indicated ligands for indicated times. After stimulation, cells were lysed, and samples of equal volume from the lysates were analyzed by Western blotting for the phosphorylated and total amounts of ERK as in Fig. 1. The HEK-β cell lysate from the 3rd-min isoproterenol stimulation was run in all gels (except the gel that contains that sample) as a reference and is indicated as *C* in the figure. Abbreviations and final concentrations for the ligands are the same as in Fig. 1 Shown are the representatives of at least three experiments with similar results.

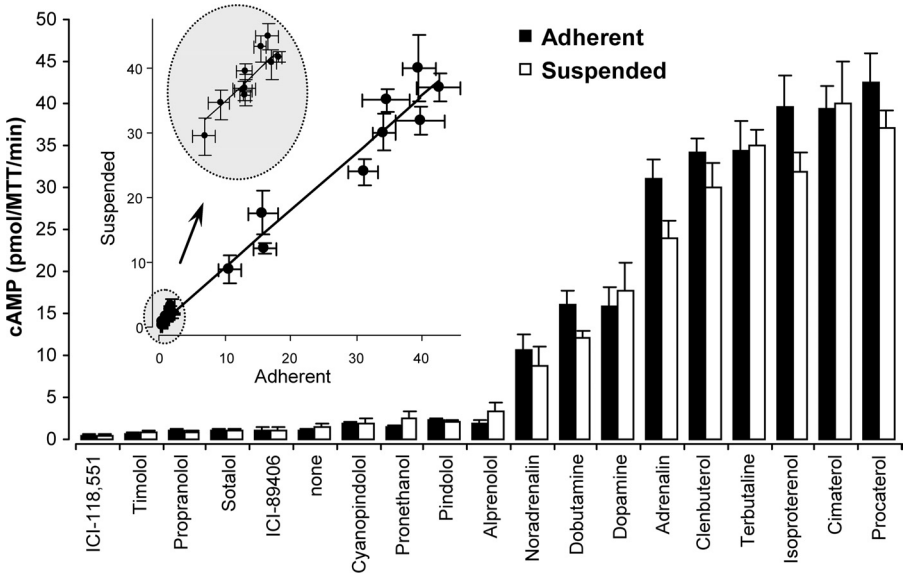


FIGURE 4. **Ligand-induced cAMP accumulation responses in HEK-β cells are not affected by the adherence state.** Adherent or suspended cells were incubated with the saturating concentrations of the indicated ligands for 5 min in the presence of 1 mM isobutylmethylxanthine. Reaction was stopped by 0.1 N HCl. Amount of accumulated cAMP was determined by radioimmuneassay and corrected with the number of viable cells as assessed by parallel MTT assays. Experiments were performed in adherent and suspended cells simultaneously. Shown are the mean ± S.E. (*error bars*) of four independent experiments run in triplicate. *Inset*, cAMP values in adherent and suspended cells are plotted against each other. Linear regression between the two responses (slope, 0.89; intercept, 0.32) is also shown by the *thick line*.

nitude of intrinsic activities for cAMP responses (except for adrenaline) was significantly different between adherent and suspended cells (Fig. 4). However, this was not the case for the ERK phosphorylation responses. Comparison of the ligand intrinsic activities for cAMP accumulation and ERK phosphorylation responses in fully confluent cells (Fig. 5, *top*) showed that there were in fact two groups of ligands that displayed functional selectivity in opposite directions: First group (G_1 in Fig. 5) was formed by the ligands that acted as strong agonists for the cAMP response but antagonists for the ERK phosphorylation (clenbuterol, cimaterol, procaterol, and terbutaline) whereas the ligands in the second group (G_2 in Fig. 5) displayed higher intrinsic activities for ERK phosphorylation than for cAMP accumulation (adrenaline and noradrenaline). The overall correlation between cAMP accumulation and ERK phosphorylation improved gradually from the adherent (100% confluent) cells to the suspended cells (Fig. 5). This improvement was due mainly to the increase in intrinsic activities of clenbuterol, cimaterol, procaterol, and terbutaline in ERK phosphorylation response in 50% confluent (Fig. 5, *middle*) and suspended cells (Fig. 5, *bottom*), whereas the positions of the other ligands in the intrinsic activity scales did not change with the adherence state of the cells. Thus, the functional selectivity of the ligands in the first group was observable conditionally, depending on whether or not cAMP could induce ERK phosphorylation.

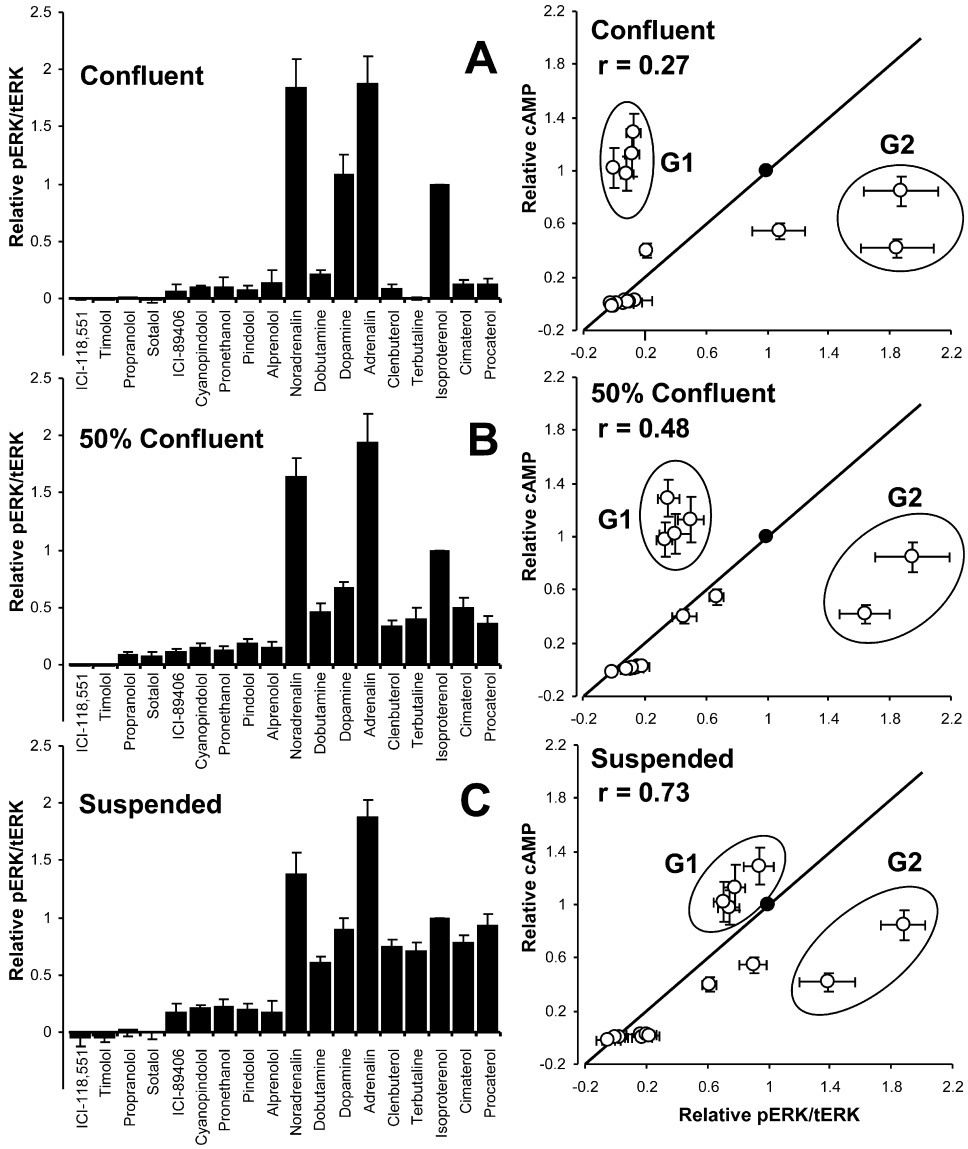


FIGURE 5. **ERK phosphorylation responses induced by β_2-AR ligands in HEK-β cells are affected by the adherence state.** Adherent 100% (*A*) or 40–50% (*B*) confluent or suspended (*C*) HEK-β cells were stimulated with the indicated ligands for 3 min. All ligands were used at saturating concentrations. After stimulation, cells were lysed, and samples of equal volume from the lysates were analyzed by Western blotting for the phosphorylated and total amounts of ERK as in Fig. 1. For each experiment, the basal pERK/tERK value was subtracted from the pERK/tERK value of each ligand to obtain ligand-induced ERK phosphorylation. The ERK phosphorylation signal for each ligand was then corrected with the isoproterenol-induced one. Data are the mean ± S.E. (*error bars*) of at least three independent determinations for each ligand for each condition. All of the ligands were assayed simultaneously for their ERK phosphorylation responses at least in three independent experiments. 6–18 independent determinations were made for the ligands that show different responses in different adherence conditions. In the *left panels*, ligands on the *x* axis are sorted with respect to their intrinsic activity for the cAMP response. In the *right panels*, ERK phosphorylation data shown in the *left panels* were plotted against the ligand-induced cAMP production. cAMP responses used in these plots were calculated from the data for attached cells in Fig. 4 by subtracting the basal value from the ligand-stimulated ones and correcting the new values with the isoproterenol-induced cAMP response. Correlation coefficients shown on the *right panels* were calculated using the corrected ERK and cAMP responses. The ligand groups that deviate from the line with slope = 1 and intercept = 0 (*thick lines*) are indicated as G_1 and G_2 in the *left panels*.

Characterization of ERK Phosphorylation Responses

PTX Sensitivity—A well established feature of the β_2-AR-mediated ERK phosphorylation by isoproterenol is its inhibition by PTX (10, 11). Therefore, we assessed the PTX sensitivity of ERK phosphorylation responses in adherent (100% confluent) and suspended cells to gain some insight into these apparently different responses. PTX treatment inhibited isoproterenol-, adrenaline-, noradrenaline-, and dopamine-induced ERK phosphorylation observed in confluent cells almost completely: The average inhibition was 79% (Fig. 6*A*) in this condition. The small but significant ERK phosphorylation response in confluent cells to dobutamine, a partial agonist for cAMP response, was also inhibited by PTX (Fig. 6*A*). In contrast, ERK phosphorylation responses induced by clenbuterol, cimaterol, procaterol, and terbutaline, as well as by forskolin in suspended cells were resistant to PTX treatment (Fig. 6*B*). Unlike in the confluent cells, PTX inhibited the isoproterenol-, adrenaline-, noradrenaline-, dopamine-, and dobutamine-induced ERK phosphorylation in suspended cells only partially (44% on the average). As noted above, the correlation between the ligand-induced cAMP and ERK responses improved in suspended cells compared with the confluent or 50% confluent cells (see the

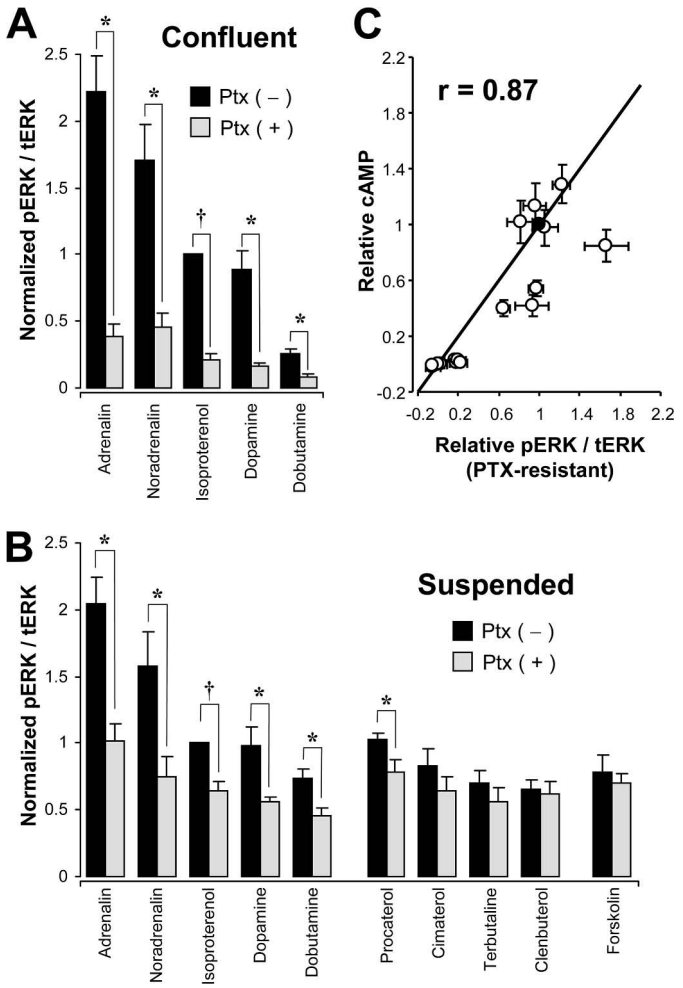


FIGURE 6. **ERK phosphorylation responses in adherent and suspended cells differ in their PTX sensitivity.** *A* and *B*, HEK-β cells were incubated overnight in serum-free culture medium with or without PTX (100 ng/ml). For the ERK phosphorylation assay in adherent cells (*A*), the medium was replaced with the serum-free fresh medium 30 min before the ligand stimulation. For the assay in suspension (*B*), cells were detached by 1 mM EDTA, centrifuged, resuspended in serum-free medium, and let rest for 30 min. Cells were then stimulated with the indicated ligands (at final concentrations given in Fig. 1) or with forskolin (10^{-5} M) or for 3 min. After stimulation, cells were lysed, and samples of equal volume from the lysates were analyzed by Western blotting for the phosphorylated and total amounts of ERK as in Fig. 1. For each experiment, the basal pERK/tERK value was subtracted from pERK/tERK value of each ligand or forskolin to obtain the stimulated ERK phosphorylation. ERK phosphorylation signal for each ligand in the absence and the presence of PTX was then corrected with the isoproterenol-induced one in the absence of PTX. Shown are the mean ± S.E. (*error bars*) of at least four independent determinations run in duplicate. *C*, PTX-resistant components of the ERK phosphorylation responses for adrenaline, noradrenaline, isoproterenol, dopamine dobutamine, procaterol, cimaterol, terbutaline and clenbuterol in *B* were used to replot the graph in *lower right panel* of Fig. 5 and to calculate the correlation coefficient shown in the figure. ERK phosphorylation data for the other ligands and the ligand-induced cAMP responses are the same as in Fig. 5. * indicates significant difference between mean values, and † indicates significant difference from 1, as assessed by Student's *t* test.

correlation coefficients in Fig. 5). It significantly improved further when the PTX-resistant components of the ERK phosphorylation responses in suspended cells were used for the same calculation (Fig. 6*C*). These results suggest that there are at least two signaling pathways that couple β_2-AR stimulation to ERK activation in HEK-β cells: (i) the PTX-sensitive pathway that presumably involves G_i/G_o type G proteins, and (ii) the PTX-resistant pathway that seems to be mediated by cAMP and inhibited by cell contact and/or cell adherence. Clenbuterol, cimaterol, procaterol, terbutaline, and forskolin seem to induce only the PTX-resistant pathway whereas isoproterenol, adrenaline, noradrenaline, dopamine, and dobutamine can induce both pathways.

Effects of PKA Inhibition—In addition to PTX, isoproterenol-induced ERK phosphorylation in β_2-AR-expressing HEK cells has also been shown to be sensitive to H89, a cAMP-dependent protein kinase (PKA) inhibitor (10, 11). This dual sensitivity has led to the currently accepted "switch" hypothesis which suggests that PKA-mediated phosphorylation of β_2-AR switches the receptor coupling from G_s to G_i and thereby initiates the ERK signaling (10, 11). Considering the potential nonspecific effects of H89 (23–25), we investigated the putative PKA involvement in the PTX-sensitive ERK phosphorylation responses in confluent cells by complementary approaches: We used two structurally different inhibitors of PKA, *i.e.* H89 or a specific, cell-permeable PKA inhibitor peptide (myr-PKI), or we knocked down the enzyme by small interfering RNAs targeting the ubiquitously expressed α and β isoforms of PKA catalytic subunits. Isoproterenol and adrenaline were chosen as the representatives of the ligands that stimulate the PTX-sensitive ERK phosphorylation. In accord with previous reports, isoproterenol-induced ERK phosphorylation response was markedly inhibited by H89 in confluent HEK-β cells (Fig. 7*A*). However, this effect was not mimicked by myr-PKI (Fig. 7*A*), although the latter was fully effective in inhibiting the phosphorylation of cAMP-responsive element-binding protein (CREB), a PKA-mediated effect (Fig. 7*B*). On the other hand, adrenaline-induced ERK phosphorylation in confluent cells was resistant to both H89 and myr-PKI (Fig. 7*A*). Moreover, an overall 75% decrease in the expression levels of α and β catalytic subunits of PKA (Fig. 7*D*), which resulted in a near-complete loss in ligand-induced CREB phosphorylation (Fig. 7*E*), did not have any effect on the ERK phosphorylation responses to either isoproterenol or adrenaline (Fig. 7*C*). Thus, among the three interventions that inhibited PKA activity, as judged by their effects on CREB phosphorylation, only H89 was effective in inhibiting the ERK phosphorylation in confluent cells. Furthermore, the latter effect was ligand-dependent. A plausible explanation for these results is that PKA is not involved in β_2-AR-mediated, PTX-sensitive ERK phosphorylation and that H89 inhibits isoproterenol-induced ERK response nonspecifically.

Involvement of β-Arrestin—β_2-AR-induced ERK phosphorylation has been shown to have a β-arrestin-mediated component that is resistant to PTX (11). Compared with the PTX-sensitive ERK response in confluent cells, a relatively long lasting time course of the ligand-induced ERK response in suspended cells (see Fig. 3) is indeed suggestive of the kinetic pattern reported for the β-arrestin-mediated ERK phosphorylation. Hence, we considered the possibility that the ligand-induced PTX-resistant ERK response in suspended cells might be mediated by β-arrestin. In the context of the present results, either differential coupling of β_2-AR to β-arrestin in different adherence conditions or modulation of β-arrestin-mediated ERK phosphorylation by adherence state would explain why the PTX-resistant ERK response could be observed conditionally. To provide more insight to these questions, we measured

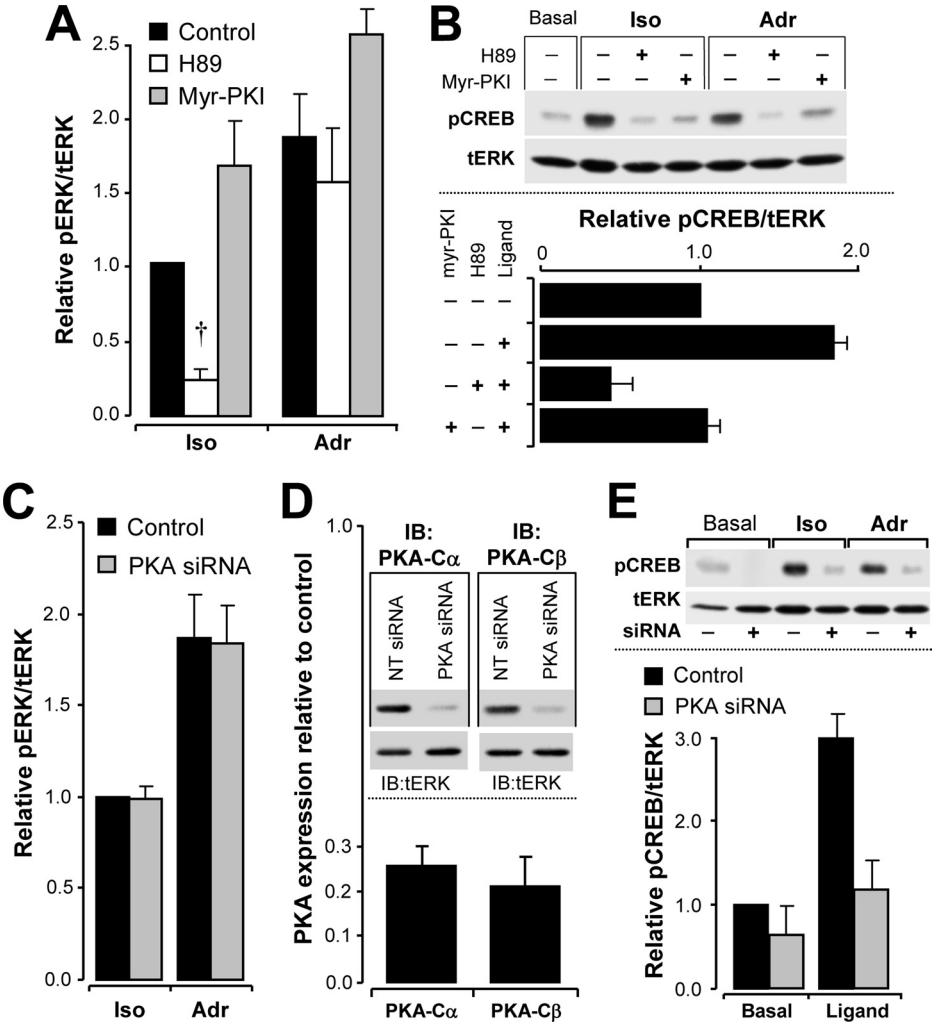


FIGURE 7. **Effects of chemical inhibition and knockdown of PKA on the PTX-sensitive ERK phosphorylation are incoherent.** *A* and *B*, adherent 100% confluent HEK-β cells were incubated in the absence or presence of PKA inhibitors H89 (10 μM) or myr-PKI (30 μM) for 20 min at 37 °C and then stimulated with the indicated ligands for 3 min. After stimulation, cells were lysed, and samples of equal volume from the lysates were analyzed by Western blotting for the phosphorylated amounts of ERK (pERK) or CREB (pCREB) and total amounts of ERK (tERK). For each experiment, the basal pERK/tERK value was subtracted from the pERK/tERK value of each ligand to obtain the stimulated ERK phosphorylation. The stimulated ERK phosphorylation signals in the absence and the presence of PKA inhibitors were then corrected with the isoproterenol-induced one in the absence of the inhibitors. Shown are the mean ± S.E. (*error bars*) of 4–8 independent experiments. *C–E*, cells at 70% confluence were transfected twice on consecutive days in 6-well plates with siRNAs targeting α (PKA-Cα) and β (PKA-Cβ) catalytic subunits of PKA (100 pmol/well, each) and nontargeting (*NT*) siRNA (200 pmol/well). One well of cells was split into 8 wells of 24-well plates at the 24th h and used for the immunoblots in *D* or the ERK (*C*) and CREB (*E*) phosphorylation assay in at the 48th h of the second transfection. The PKA subunit expression levels (*D*) and the ERK (*C*) or CREB (*E*) phosphorylation responses are the mean ± S.E. of four independent experiments. Ligand-induced pCREB/tERK values in all conditions were corrected with the basal value. Corrected pCREB/tERK values obtained for isoproterenol and adrenaline were pooled for each condition and shown as ligand-induced CREB phosphorylation in the graphs in *B* and *E* because no significant difference was found between the ligands in inducing CREB phosphorylation in the presence or absence of PKA inhibition. Representative blots are shown in *B*, *D*, and *E*. Abbreviations and final concentrations for the ligands are the same as in Fig. 1. * indicates significant difference between mean values, and † indicates significant difference from 1, as assessed by Student's *t* test.

β_2-AR-β-arrestin interaction directly in the presence of those ligands that showed differential behavior in terms of ERK phosphorylation in adherent and suspended cells. We used a resonance energy transfer assay to assess β_2-AR-β-arrestin interaction, where β_2-AR-rLuc acted as the donor, and GFP-β-arrestin2 as the acceptor. Fig. 8*A* shows that ligands such as clenbuterol, cimaterol, and terbutaline induced receptor-arrestin interaction (albeit weak compared with receptor G_s interaction) in adherent cells where they did not induce any ERK phosphorylation at all. This observation may suggest that the efficiency of arrestin-mediated ERK phosphorylation increases in suspended cells compared with the adherent cells, which may then explain the emergence of PTX-resistant ERK responses to clenbuterol, cimaterol, terbutaline, and procaterol in suspended cells. However, the fact that clenbuterol, among the ligands mentioned above, possesses too weak an ability to couple the receptor to arrestin (Fig. 8*A*) despite its strong action on ERK phosphorylation in suspended cells, weakens the latter possibility. Nevertheless, the possibility that cell detachment increases the ligand-induced β_2-AR-β-arrestin interaction, which in turn compensates for the difference between clenbuterol and the other ligands in terms of receptor-arrestin coupling, still remains. To test the latter possibility, we compared ligand-induced β_2-AR-β-arrestin coupling in adherent and suspended cells using clenbuterol as an extreme case (as explained above) and adrenaline as reference, which behaved

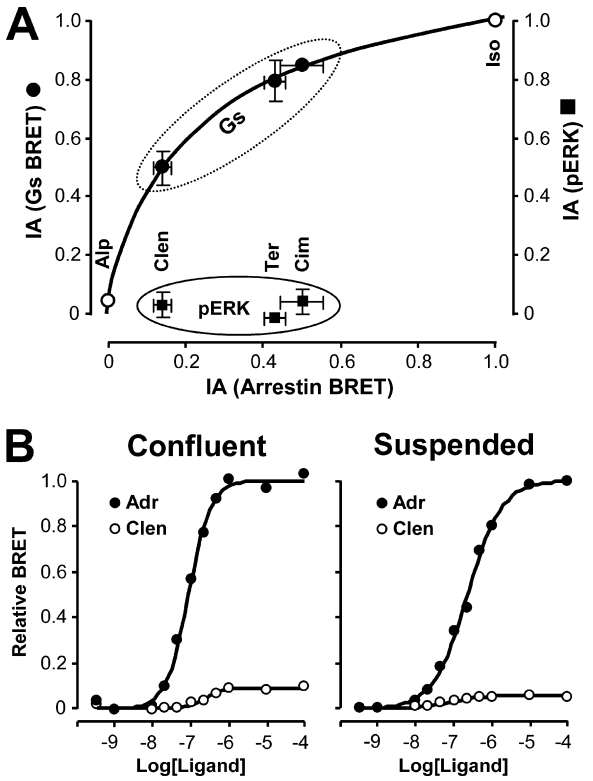


FIGURE 8. **Ligand-induced β_2-AR-β-arrestin interaction in HEK-293 cells.** *A*, ligand intrinsic activities (fractional E_{max} minus basal) were determined from concentration-response curves of indicated ligands or using a single saturating ligand concentration (alprenolol, 10 μM). The data are means ± S.E. (*error bars*) of three to six experiments. *Alp*, alprenolol; *Clen*, clenbuterol; *Ter*, terbutaline; *Cim*, cimaterol; *Iso*, isoproterenol. *B*, concentration response-curves for adrenaline (*Adr*) or clenbuterol (*Clen*)-induced receptor-arrestin interaction measured in adherent or suspended cells. Data are expressed as fraction of the maximal effect of adrenaline after subtraction of base-line signals. Note that the relative effect of clenbuterol is not significantly different between two experimental conditions.

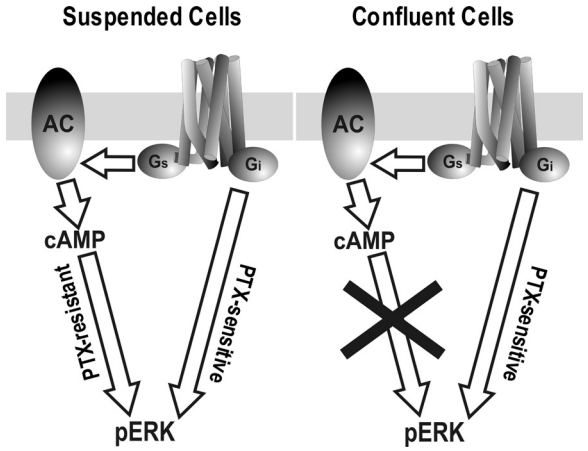


FIGURE 9. **Schematic representation of the β_2-AR-induced ERK phosphorylation in HEK cells.** At least two pathways, one PTX-resistant and the other PTX-sensitive, couple β_2-AR to ERK phosphorylation. G_s/cAMP or G_i/G_o type of G proteins mediates PTX-resistant or PTX-sensitive pathways, respectively. PTX-resistant pathway can be stimulated by all of the β_2-AR ligands that increase intracellular cAMP, whereas the PTX-sensitive pathway can be activated only by isoproterenol, adrenaline, noradrenaline, dopamine, and dobutamine. The cAMP-mediated pathway is apparently inhibited by cell contact and thus, can be activated only when cell contact is decreased as when the cells are brought to suspension. The G_i/G_o-mediated pathway, on the other hand, does not seem to be modulated by cell contact.

similarly in adherent and suspended cells in terms of ERK responses. The results of this experiment are shown in Fig. 8*B*, where no difference was observed in clenbuterol-induced β_2-AR-β-arrestin coupling in adherent and suspended cells. Therefore, we concluded that the conditional emergence of the PTX-resistant ERK responses in the presence of these ligands can hardly be attributed to a differential involvement of arrestin (if at all) in ERK responses in adherent and suspended cells.

DISCUSSION

We showed that the intrinsic activities of β_2-AR ligands for stimulating cAMP production and ERK phosphorylation responses in HEK cells were not correlated. The lack of correlation resulted mainly from the discrepancy between the intrinsic activities of two groups of ligands for these two responses: The first group consisted of clenbuterol, cimaterol, procaterol, and terbutaline, which acted as full agonists for cAMP production but displayed weak or null effect on ERK phosphorylation. The second group, on the other hand, comprised adrenaline and noradrenaline which displayed higher intrinsic activities for the ERK phosphorylation than for the cAMP response. Thus, both groups behaved as functionally selective ligands. The functional selectivity of the first group was observable only in adherent cells when confluence was approximately 100%. When cell-cell contact was minimized either by decreasing the density of the adherent cells or by bringing the cells into suspension, the first group of ligands gained the ability to stimulate ERK phosphorylation without a change in their effect on cAMP production. In contrast, selectivity of the second group was independent from the adherence state of the cells.

The simplest model that explains the present observations is schematized in Fig. 9. Taken as a whole, the data suggest that there exist at least two pathways in HEK cells that couple β_2-AR activation to ERK phosphorylation. One is the PTX-resistant pathway which can be stimulated by all the β_2-AR ligands that increase intracellular cAMP. This pathway is apparently inhibited by cell-cell contact and thus, can be activated only when cell-cell contact is decreased. The second pathway is sensitive to PTX, can be activated only by isoproterenol, adrenaline, noradrenaline, dopamine, and dobutamine, and is not modulated by cell contact. The ERK phosphorylation response to the latter five ligands is mediated solely by the PTX-sensitive pathway in confluent cells, but contributed by both pathways in suspended cells, as the inhibition by PTX for these ligands is almost complete in confluent cells but only partial in suspended cells. Intracellular cAMP increase does not seem to be directly involved in the β_2-AR-activated, PTX-sensitive pathway because (i) this pathway can be induced by some but not all of the β_2-AR ligands that increase intracellular cAMP to a comparable extent, and (ii) the resulting ERK phosphorylation is observable in confluent cells where forskolin and db-cAMP do not cause a measurable ERK phosphorylation. PTX-resistant ERK phosphorylation, on the other hand, seems to be mediated by cAMP because (i) for all of the ligands tested in this study, the intrinsic activities for β_2-AR-induced cAMP increase and PTX-resistant ERK phosphorylation responses are correlated, and (ii) there is also a perfect correlation between the observability of forskolin- or db-cAMP-induced and β_2-AR-induced PTX-

resistant ERK phosphorylation, depending on the adherence state of the cells. It is this cAMP-mediated component of ERK phosphorylation that is apparently under inhibitory control of cell-cell contact in HEK cells.

In GPCR systems, PTX sensitivity is a distinctive feature of G_i/G_o-mediated cellular responses (26). Stimulation by isoproterenol has been shown to cause GTP loading of G_i protein in the membranes of HEK-293 cells overexpressing β_2-AR, indicating that the receptor could interact with G_i (10). Using a more direct resonance energy transfer-based method, Casella *et al.* (22) have shown that isoproterenol-bound β_2-AR could indeed interact with PTX-sensitive G proteins. Therefore, we may say that in adherent cells, intracellular cAMP increase and PTX-sensitive ERK phosphorylation responses are good indicators of G_s and G_i/G_o activation, respectively, by β_2-AR stimulation. Hence, the present results suggest that, among the β_2-AR ligands that induce G_s coupling, only some are able to couple β_2-AR to G_i/G_o efficiently. The latter observation provides a clear example of a functional selectivity, a concept based on the fact that different ligands can activate diverse pathways of receptor signaling differentially. Such a ligand-dependent selectivity in receptor signals has generally been accepted as a new pharmacological dimension in GPCR-mediated signaling. In the present observations, it is worth noting that those ligands that are apparently able to couple the receptor to G_i/G_o (in addition to G_s) all contain an intact catechol ring in their structure, whereas those that can activate only G_s do not. Thus, we speculate that the interactions of catecholic OH groups in ligands with the receptor conserved serine and asparagine (Ser-204, Ser-207, and Asn-293) residues (27, 28) may be a critical factor that determines the observed G protein selectivity.

The functional selectivity that β_2-AR ligands clearly possess is blunted when the inhibitory control of cell contact on the cAMP-mediated ERK phosphorylation is relieved because in this condition all ligands that are able to increase cAMP also cause ERK phosphorylation, which eventually masks the otherwise existing "bias" of ligands. An important implication of this result is that the inherent bias of ligands in coupling a GPCR to different transducers may not always be revealed as a functional selectivity when there is a cross-talk between the signaling pathways activated by the same receptor. To our knowledge, this is the first example of such a "conditional observability" of functional selectivity.

As noted in the Introduction, the mechanisms of ERK phosphorylation mediated by β_2-AR and other G_s-coupled receptors have not been fully understood. In the so-called switch model (10), sensitivity of β_2-AR-induced ERK phosphorylation in HEK-293 cells to both PTX and PKA inhibitor H89 has been explained in the context of a single pathway that involves both a G_i/G_o type of G protein and cAMP. This model suggests that PKA-mediated phosphorylation of the receptor switches the receptor coupling from G_s to G_i, which, in turn, mediates the ERK phosphorylation. There, an indirect role is attributed to cAMP in ERK phosphorylation, and the ability of forskolin to cause ERK phosphorylation remains unexplained. As discussed above, the present results as a whole cannot be explained by a single pathway. The data, instead, suggest that in HEK cells, β_2-AR stimulates ERK phosphorylation by at least two pathways, one of which seems to be directly mediated by cAMP. However, the PTX-sensitive component, which should be cAMP-independent for the reasons explained above, is sensitive to PKA inhibition by H89 in the present experimental system as well. This brings the question as to how a seemingly cAMP-independent response can be sensitive to PKA inhibition. The answer, we believe, lies, in the contradictory results that we obtained by using different strategies to inhibit PKA. Inhibition of PTX-sensitive component of the isoproterenol-induced ERK response by H89 was not mimicked by either myr-PKI or PKA knockdown, suggesting a nonspecific mechanism rather than PKA inhibition for the effect of H89. Therefore, we may say that PKA is not involved in the PTX-sensitive component of the β_2-AR-induced ERK phosphorylation. Furthermore, the observation that the isoproterenol-induced but not the adrenaline-induced ERK phosphorylation was sensitive to H89 suggests that the H89 sensitivity is not a general property of β_2-AR-induced ERK phosphorylation but a ligand-dependent phenomenon. The latter observation reproduces the results of previous studies conducted by using isoproterenol (10, 11) or adrenaline (18, 29) and, by itself, is an interesting phenomenon which may worth further investigation.

Cell adherence to the extracellular matrix and contacts among neighboring cells seem to be crucial factors affecting the cellular responses to various stimuli, particularly those that are involved in cell differentiation and proliferation (30). Modulatory effects of cell adherence on ERK activation have been investigated mostly in the context of the canonical receptor tyrosine kinase-Ras-ERK signaling cascade. In those studies, ERK phosphorylation responses to receptor tyrosine kinases were found to be augmented considerably in adherent cells compared with the cells in suspension (31–33). Although the underlying mechanisms are not clear, available data suggest that integrin-dependent cell adherence to extracellular matrix, particularly to fibronectin, increases the efficiency of receptor tyrosine kinase activation (34) and/or signaling between Ras and its downstream effector Raf (32). Although relatively few, studies on the impact of cell adherence on GPCR signaling also indicate an enhancing effect of integrin-mediated adherence on ERK activation responses to various GPCR ligands, including bombesin, lysophosphatidic acid, and ATP (35, 36). Thus, the modulatory effect of cell adherence that we observed for the β_2-AR-induced and presumably cAMP-mediated ERK phosphorylation is in the opposite direction of what has generally been reported for receptor tyrosine kinases or other GPCRs, except for the serotonin $5HT_{1D}$ receptor in LTK fibroblasts (37) and angiotensin ATII receptor in human mesangial cells (38). Moreover, cell-cell contact rather than adherence to extracellular matrix seems to be the modulating factor on β_2-AR-induced ERK phosphorylation; first because we did not use any matrix coating in our experiments, and second, but more importantly, decreasing the density of adherent cells and suspending the cells had qualitatively similar effects on ERK responses. Cell-cell contact, in turn, is mediated by the cadherin family of proteins which have also been shown to modulate mitogenic signaling (30, 39, 40). Thus, a cadherin-mediated mechanism rather than an integrin-mediated one seems to be a more likely candidate for modulating the cAMP-mediated

component of β_2-AR-induced ERK phosphorylation. However, at this point, how and at which point of the pathway the cell-cell contact interferes with the cAMP-mediated ERK activation remains as a novel and interesting question that arises from the present study.

Finally, it would be prudent to mention that all of the results and conclusions of the present study should be considered in the context of the present experimental system. The modulatory effect of cell contact on the cAMP-mediated ERK phosphorylation, and thus the conditional nature of ligand selectivity, may not be observed in all cells, as the cAMP-mediated ERK phosphorylation is well known to be cell type-dependent. Therefore, the results of the present study should not be generalized to other GPCRs or cell systems without further experimentation. However, the latter fact can be considered as a good reason to investigate different GPCRs in different conditions to better understand the mechanisms of GPCR-mediated ERK phosphorylation and regulation of GPCR-mediated signaling in general.

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