0026-895X/96/051376-12$3.00/0

MOLECULAR PHARMACOLOGY, 50:1376–1387 (1996).

Coupling Efficiencies of Human α_1-Adrenergic Receptor Subtypes: Titration of Receptor Density and Responsiveness with Inducible and Repressible Expression Vectors

TRACEY L. THEROUX, TIMOTHY A. ESBENSHADE, RICHARD D. PEAVY, and KENNETH P. MINNEMAN

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Received March 20, 1996; Accepted July 25, 1996

SUMMARY

We compared the efficiencies with which human α_1-adrenergic receptor (AR) subtypes activate inositol phosphate (InsP) formation and increase intracellular Ca^{2+} in transfected cell lines. Expression of human α_{1a}-, α_{1b}-, and α_{1d}-AR cDNAs under the repressible control of anhydrotetracycline in human embryonic kidney (HEK) 293 cells, which normally express no α_1-ARs, was used to compare responses to norepinephrine (NE) at different receptor densities. Maximal NE-stimulated InsP formation was found to increase with increasing density of each subtype, whereas basal levels and responses to sodium fluoride did not change. A comparison of multiple subclones over equivalent ranges of receptor expression showed that activation of each subtype resulted in different maximal responses ($\alpha_{1a} > \alpha_{1b} > \alpha_{1d}$) in HEK 293 cells. Analogous studies were carried out in human SK-N-MC cells, which normally express low levels of all three α_1-AR subtypes, using an isopropyl-β-D-thiogalactoside-inducible expression system. Induction with isopropyl-β-D-thiogalactoside increased the density of individual α_1-AR subtypes by 4–6-fold over the level of endogenous expression. Increased expression of each of these subtypes in SK-N-MC cells did not alter the EC_{50} value for NE in stimulating InsP formation or releasing $[Ca^{2+}]_i$ but did increase maximal responses to NE. Similar to our findings in HEK 293 cells, a comparison of responses at similar expression levels in SK-N-MC cells showed different maximal responses stimulated by each subtype, for both InsP ($\alpha_{1a} > \alpha_{1b} \geq \alpha_{1d}$) and $[Ca^{2+}]_i$ ($\alpha_{1a} > \alpha_{1b} > \alpha_{1d}$) responses. These studies show that agonist-occupied human α_1-AR subtypes have different efficiencies in activating phospholipase C in human cell lines. In both HEK 293 and SK-N-MC cells, α_{1a}-ARs couple most efficiently, whereas α_{1d} -ARs couple very poorly.

Responses to NE and epinephrine are mediated through three families (i.e., α_1, α_2, and β) of AR subtypes (1). Each family acts through distinct G protein-mediated pathways to activate a separate signal transduction system. α_1-ARs increase intracellular Ca^{2+}, α_2-ARs inhibit adenylate cyclase, and β-ARs stimulate adenylate cyclase. There are at least three closely related subtypes within each family, each of which seems to activate the same signaling system. It is not yet clear whether closely related subtypes within a family are equally capable of activating such common signaling mechanisms or whether different subtypes might do so with different efficiencies.

There are three α_1-AR subtypes (i.e., α_{1A}, α_{1B}, and α_{1D}) encoded by separate genes (i.e., α_{1a}, α_{1b}, and α_{1d}) (2–7). α_1-ARs are involved in a variety of important functions, including smooth muscle contraction, neurotransmission, and control of cell growth. These receptors seem to act through G proteins of the G_q and/or G_h families (8, 9) to increase InsP production through activation of PLC and increase $[Ca^{2+}]_i$ via release of stored Ca^{2+} and/or activation of voltage-dependent Ca^{2+} channels (2, 10, 11). All three recombinant α_1-AR subtypes can increase InsP production and $[Ca^{2+}]_i$ (12–15), although some experiments suggest that there may be differences in the ability of each subtype to activate these responses (12, 13).

In this study, we compared the relative coupling efficiencies of human α_1-AR subtypes in increasing both [^{3}H]InsP formation and $[Ca^{2+}]_i$. Because the coupling efficiency of the agonist-occupied receptor should be reflected in its ability to activate the signaling response at a particular receptor density, we measured changes in agonist-stimulated second messenger responses over a range of receptor expression. We stably transfected two different human cell lines with inducible or repressible eukaryotic expression systems containing

This work was supported by National Institutes of Health Grant NS32706 (K.P.M.).

ABBREVIATIONS: NE, norepinephrine; AR, adrenergic receptor; PLC, phospholipase; PCR, polymerase chaine reaction; ANTET, anhydrotetracycline; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; InsP, inositol phosphate; IPTG, isopropyl-β-D-thiogalactoside; HEK, human embryonic kidney; tTA, tetracycline *trans*-activator; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

sequences encoding the three human α_1-AR subtypes (16–18). A tetracycline-repressible expression system (19) was used in HEK 293 cells, whereas an IPTG-inducible vector system (20) was used in SK-N-MC human neuroblastoma cells. HEK 293 cells express no detectable α_1-ARs, whereas SK-N-MC cells endogenously express low levels of all three α_1-AR subtypes (10, 18). G protein-coupled receptor signaling mechanisms have been well characterized in both cell lines (21, 22). An examination of agonist-stimulated responses in these two human cell lines over a range of different receptor densities allows a direct comparison of the efficiencies with which each of the recombinant α_1-AR subtypes activates a common signaling pathway.

Experimental Procedures

Materials. SK-N-MC and HEK 293 cells were obtained from American Type Culture Collection (Rockville, MD). The cDNA for the human α_{1a}-AR and the gene fusion construct for the human α_{1b}-AR were generously provided by Dr. G. Tsujimoto (National Children's Hospital, Tokyo, Japan) and by Dr. R. Graham (Victor Chang Institute, Sydney, Australia), respectively. The tetracycline-repressible expression vectors pUHD 15–1, pUHD 10–3, and pUHC 13–3 were kindly provided by Dr. Hermann Bujard, Zentrum fur Molekulare Biologie der Universitat Heidelberg (Heidelberg, Germany). Lac-Switch vector system was from Stratagene (La Jolla, CA); phentolamine mesylate was from Ciba-Geigy (Summit, NJ); hygromycin B was from Boehringer-Mannheim (Indianapolis, IN); BE 2254 ([2-β-(4-hydroxyphenyl)ethylaminomethyl]-tetralone) was from Beiersdorf AG (Hamburg, Germany); [^{3}H]inositol (20–40 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO); carrier-free Na^{125}I was from Amersham (Arlington Heights, IL); ANTET was from Acros (Geel, Belgium); Fura-2/AM was from Molecular Probes (Eugene, OR); fetal bovine serum, geneticin, and trypsin/EDTA were from GIBCO BRL (Gaithersburg, MD); and digitonin, (−)-norepinephrine bitartrate, yohimbine hydrochloride, sodium fluoride, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and all other chemicals were from Sigma Chemical (St. Louis, MO).

Construction of hygromycin-resistant pUHD 15–1 plasmid. To select for transfected HEK 293 subclones, we introduced the hygromycin-B/phosphotransferase gene into the pUHD 15–1 plasmid (19). Primers were designed to produce a PCR product encoding the entire hygromycin-B/phosphotransferase gene from pCEP4 (InVitrogen, San Diego, CA) with *Bam*HI (GIBCO BRL) restriction sites at both ends. The cDNA sequences of the primers were sense (upstream from the promoter site), 5′-CGGGATCCATGCCCTGCTTCATCC-3′, and antisense [downstream from the poly(A)$^+$ tail], 5′-CGGGATCCAGTCGTGGACCAGACC-3′. The cDNA amplification product was predicted to be 1670 bp long.

cDNA for the hygromycin-B/phosphotransferase gene was amplified by PCR in a 100-μl reaction mixture containing 2.5 mM Tris·HCl, pH 8.9; 5 mM KOAc; 0.2 mM $MgSO_4$; 200 μM concentration each of dATP, dCTP, dGTP, and dTTP; 0.5 μM concentration of each primer; 1.5 units of *Taq* polymerase (Life Technologies, Gaithersburg, MD); and 0.003 unit of Vent polymerase (New England Biolabs, Beverly, MA). The PCR was performed with a Biometra TRIO-Thermoblock with the following cycle parameters: 30 cycles of 1 min at 94°, 1 min at 43°, and 5 min at 72°. A single PCR product of ~1.7 kb was obtained, representing the full-length hygromycin-B/phosphotransferase gene. The PCR reaction mixture was passed through a size-exclusion resin (PCR Magic Prep, Promega, Madison, WI), digested with *Bam*HI, and ligated into the unique *Bam*HI site of pUHD 15–1, distal to the gene encoding the tTA protein.

Preparation of expression vectors. The full-length human α_{1a}-AR cDNA (16), the α_{1b}-AR gene fusion construct (17), and the α_{1d}-AR cDNA/gene fusion construct (18) were cloned into the multiple cloning site in the pUHD 10–3 operator vector of the tetracycline-repressible expression dual-plasmid system. The full-length cDNAs for these subtypes were also subcloned into the operator vector (pOPRSVICAT) of the inducible Lac-Switch system (Stratagene), modified as described previously (23).

Cell culture. SK-N-MC (10) and HEK 293 (15) cells were propagated in 75-cm^2 flasks in a humidified 7% CO_2 incubator in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 100 mg/liter streptomycin and 10^5 units/liter penicillin and supplemented with 10% fetal bovine serum.

Transfection and selection. HEK 293 cells were cotransfected by calcium phosphate precipitation with the pUHD 15–1 vector (3 μg/100-mm plate) and pUHD 10–3 vector containing one of the three human α_1-AR cDNA clones (3 μg/100-mm plate). The cells were allowed to recover for 3 days and subjected to selection pressure with 25 μg/ml hygromycin. Resistant cells were propagated and individual subclones were isolated by limiting dilution plating. Subclones were screened for receptor density and stability of receptor expression and propagated in the continued presence of hygromycin. A total of six subclones (two of each subtype) were chosen for further studies. Receptor expression was repressed by the addition of a single dose of 1 μg/ml ANTET from 3 hr (for minimal repression) to 7 days (for lowest expression levels). The medium was not changed for the duration of ANTET treatment.

SK-N-MC cells were first transfected with the Lac-Switch repressor vector (p3′SS) by calcium phosphate precipitation, and cells resistant to 200 μg/ml hygromycin were selected and propagated. These resistant cells were then transfected with the individual operator vectors containing one of the human α_1-AR subtypes. After recovery, the cells were exposed to geneticin (400 μg/ml) for several weeks to select resistant cells, which were propagated in the presence of both antibiotics. Subclones expressing each of the different α_1-AR subtypes were obtained through screening for cell lines that exhibited low constitutive and high inducible levels of α_1-AR binding sites. Cells for radioligand binding, [^{3}H]InsP formation, and Fura-2 measurements of $[Ca^{2+}]_i$ were plated at lower (10%) antibiotic concentrations before assays.

^{125}I-BE 2254 binding. ^{125}I-BE 2254 binding was performed in membrane preparations as described previously (10).

Luciferase assay. HEK 293 cells were pretreated with tetracycline for 2 days before transfection with 3 μg of pUHD 15–1 and pUHC 13–3 (identical to pUHD 10–3 with the luciferase gene replacing the multiple cloning site). The luciferase assay used was a modification of the method described by deWet *et al.* (24). Cells were washed twice with phosphate-buffered saline and lysed for 10 min at 22° by the addition of 0.2 ml of lysis buffer (25 mM Tris-PO_4, 2 mM dithiothreitol, 2 mM EGTA, 10% glycerol, 1% Triton X-100, pH 7.8). The lysates were scraped, transferred to microcentrifuge tubes, and centrifuged for 5 min. A 50-μl aliquot of the supernatant was added to 350 μl of assay buffer (25 mM Tris-PO_4, 20 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM dithiothreitol, pH 7.8) in a luminometer (model TD-20e, Turner Designs, Sunnyvale, CA); 100 μl of 1 mM luciferin was injected, and integrated peak luminescence was measured over a 10-sec window after a 5-sec delay.

InsP formation. Accumulation of [^{3}H]InsP was determined in confluent 35-mm dishes. Cells were prelabeled with *myo*-[^{3}H]inositol (2 μCi/plate) for 3–4 days, and the production of total [^{3}H]InsPs was determined as described previously (10).

$[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was determined with Fura-2 as described previously (10). In brief, cell suspensions were prepared (3–4 × 10^6 cells/ml) and loaded for 15 min with 1 μM Fura-2/AM. The cells were then washed and resuspended in balanced salt solution [130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, with 0.1% bovine serum albumin (2 × 10^6 cells/ml)], and 3-ml aliquots were used for each assay. Fura-2 measurements were performed in a Perkin-Elmer (Beaconsfield, Buckinghamshire, UK) LS 50 luminescence spectrofluorometer as described previously.

Determination of surface receptors by whole-cell binding. To ensure that expressed receptor-binding sites were localized to the external cell membrane, whole-cell binding approaches were used (25). Comparison of the displacement of radioligand from whole-cell preparations by the hydrophilic agonist NE and the hydrophilic antagonist phentolamine allows conclusions to be made about the proportion of receptor-binding sites that are located on the external cell surface.

Cells were harvested as described for $[Ca^{2+}]_i$ determinations, and suspended in lithium-containing Krebs-Ringer bicarbonate buffer. ^{125}I-BE binding was performed in lithium-Krebs-Ringer bicarbonate buffer in a final volume of 0.25 ml containing suspended cells, 50 pM ^{125}I-BE, and drugs. Cells were incubated for 16 hr on ice or 20 min at 37°, and incubations were stopped by dilution and filtration as described previously for membranes (10). Total specific binding was defined as binding displaced by 10 μM phentolamine. Surface accessible binding was defined as binding displaced by 1 mM NE. The difference between surface accessible and total binding was presumed to be due to internalized binding sites (25).

Preliminary studies were performed on HEK 293 cells transfected with constitutively active expression vectors containing the bovine α_{1a}, hamster α_{1b}, and rat α_{1d} subtypes (15). In these experiments, NE displaced whole-cell specific ^{125}I-BE binding (three experiments) with $-\log IC_{50}$ values of 4.7 ± 0.2, 4.2 ± 0.1, and 6.7 ± 0.1 on ice and 4.6 ± 0.3, 3.9 ± 0.1, and 6.1 ± 0.1 at 37° for the α_{1a}, α_{1b}, and α_{1d} subtypes, respectively. The proportion of specific binding displaced by 1 mM NE was 87 ± 7%, 96 ± 6%, and 92 ± 3% on ice and 43 ± 5%, 87 ± 5%, and 69 ± 3% at 37° for the α_{1a}, α_{1b}, and α_{1d} subtypes. In HEK 293 cells transfected with the pUHD vectors containing the human subtypes (not repressed), 1 mM NE displaced total whole-cell specific binding (defined by 10 μM phentolamine) on ice by 93 ± 4%, 88 ± 3%, and 85 ± 11% for α_{1a}, α_{1b}, and α_{1d} subtypes, respectively. These data suggest that most of the measurable binding sites in these cells are located on the cell surface.

Results

Characterization of a tetracycline-repressible expression system in HEK 293 cells. The tetracycline-repressible expression system described by Gossen and Bujard (19) was used to control α_1-AR expression in HEK 293 cells. In this two plasmid system, one vector constitutively expresses a tTA fusion protein that binds with high affinity to the promoter of the second vector and increases transcription of the cDNA in the second vector. Transcription is suppressed by the addition of tetracycline, which binds to the tTA protein and prevents its actions. To examine this system in HEK 293 cells, we performed transient transfections with a luciferase reporter gene, and modulation of luciferase activity by tetracycline treatment was examined. As previously reported in other cell lines (19), tetracycline caused >100-fold repression of luciferase activity, with an EC_{50} value of 0.01 μg/ml and maximal repression at 1.0 μg/ml (data not shown).

ANTET, a close structural analog of tetracycline that is 100-fold more potent (26), was used in subsequent experiments. Fig. 1 shows the time course for inhibition of α_1-AR expression by exposure to ANTET in a subclone of HEK 293 cells (no. 17) transfected with this vector system containing the cDNA for the human α_{1a}-AR. Incubation of cells with a single high dose of ANTET (1 μg/ml) caused a 75–80% decrease in receptor density with a $t_{1/2}$ of ~7 hr. Maximal inhibition was observed after 24-hr ANTET exposure and was maintained for ≤1 week (data not shown). Similar effects were observed in the other subclones studied and when tetracycline itself was used for repression, although the time course and maximal inhibition showed some variability (data not shown).

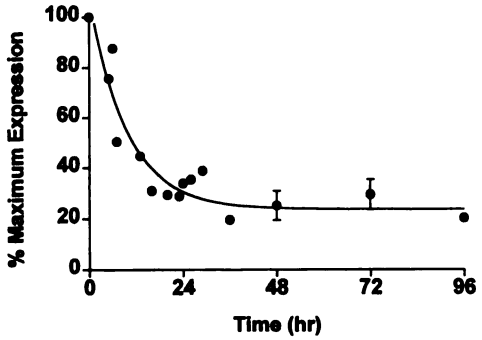

Fig. 1. α_1-AR expression is repressed in a time-dependent manner by ANTET in transfected HEK 293 cells. ANTET (1 μg/ml) was added to 100-mm plates of an α_{1a}-expressing subclone (no. 17) for the indicated time intervals. Receptor density (B_{max}) was determined by nonlinear regression analysis of saturation of ^{125}I-BE binding to membrane preparations. The results are expressed as a percentage of B_{max} in control, untreated plates (4379 ± 120 fmol/mg of protein). Data are mean ± standard error from three separate experiments.

In contrast to the >99% repressibility of luciferase activity in HEK 293 cells, ANTET treatment reduced α_1-AR expression by only 50–80% in plates prepared from the same culture passage (data not shown). Similar observations were reported by Howe *et al.* (27), who used this system to express the glutamate receptor subunit 6 in HEK 293 cells. However, many of our subclones gradually lost receptor expression with increasing passage number. A comparison of data from multiple passage numbers, multiple times of ANTET exposure, and multiple subclones allowed us to study a wider range of receptor densities than was possible with ANTET repressibility alone.

To ensure that changes in agonist-induced responses were due to changes in receptor expression and not to other effects of prolonged ANTET exposure, several parameters were compared in treated and untreated cells. There were no visible changes in the shape, growth, or clumping of cells during ANTET exposure (data not shown). Exposure to ANTET (1 μg/ml) for ≤4 days did not alter total protein levels, basal InsP formation, or InsP formation stimulated by 20 mM NaF in these cells (data not shown). Similar data were also obtained for the other subclones examined.

Relationship between expression levels of each subtype and NE-stimulated InsP formation in HEK 293 cells. We compared the magnitude of NE-stimulated InsP formation with the expression level (B_{max}) of each α_1-AR subtype to determine the relative coupling efficiencies with which each subtype activates a common second messenger system. Transfected HEK 293 subclones expressing each subtype were exposed to a single dose of 1 μg/ml ANTET at six time points (from 3 hr to 7 days) before assays to produce a range of receptor expression levels. Maximal NE-stimulated InsP formation (stimulation by 100 μM NE) and the *B*max for ^{125}I-BE binding determined by saturation analysis were measured in parallel plates. These two parameters were compared to assess the changes in [^{3}H]InsP formation associated with an increase in receptor expression levels.

In individual experiments, we found striking correlations between NE-stimulated InsP formation and increasing receptor density (Fig. 2). The magnitude of the response

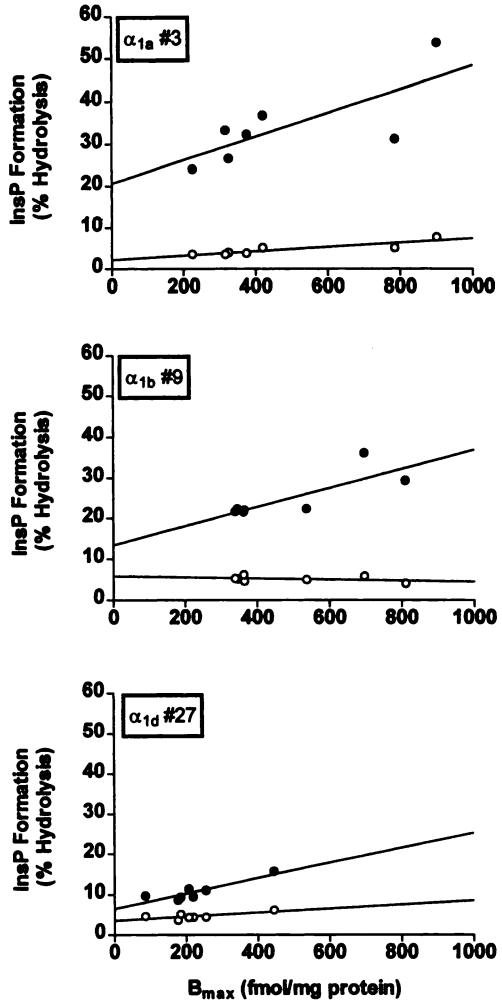

Fig. 2. Effect of density of α_1-AR subtypes on NE-stimulated InsP formation in subclones of transfected HEK 293 cells from individual experiments. A range of receptor densities was obtained by incubating subclones expressing the α_{1a}-AR (subclone no. 3, *top*), α_{1b}-AR (subclone no. 9, *middle*), and α_{1d}-AR (subclone no. 27, *bottom*) subtypes in the presence or absence of ANTET (1 μg/ml) for 3 hr to 7 days. Duplicate plates were then incubated with (●) or without (○) 100 μM NE for 1 hr, and [^{3}H]InsP formation measured as described in Experimental Procedures. Data are expressed as percentage of hydrolysis of total ^{3}H-labeled phospholipid and represent the mean of duplicate determinations. Saturation of ^{125}I-BE binding in membranes prepared from cells treated identically was analyzed by nonlinear regression to determine receptor density (B_{max}). Best fit lines of the correlations were determined by linear regression and are extrapolated to cover the entire range of receptor expression of the three different subclones. The slope of the regression line for NE is significantly different from zero for the α_{1b} (p = 0.026) and α_{1d} (p = 0.008) subtypes and is very close to significance (p = 0.052) for the α_{1a} subtype. The slope of the regression line in the absence of NE is not significantly different from zero for any of the three subtypes.

seemed to depend on both receptor density and the identity of the subtype expressed. NE-stimulated InsP formation is most robust in HEK 293 cells expressing the α_{1a}-ARs, intermediate in cells expressing the α_{1b}-ARs, and low in cells expressing the α_{1d}-ARs. Similar patterns were observed in additional experiments with the same subclones (three experiments) and in experiments with other subclones (see below), although the levels of basal and agonist-stimulated responses often varied substantially among experiments.

The relationship between NE-stimulated InsP formation and receptor density for each α_1-AR subtype is also observed in data compiled from multiple experiments with two different subclones (Fig. 3). Again, differences among the three subtypes are apparent, with robust responses in α_{1a}-AR-expressing cells, intermediate responses in α_{1b}-AR-expressing cells, and weak and variable responses in α_{1d}-AR-expressing cells. Although there is a positive correlation between the magnitude of the InsP response and receptor density for both α_{1a}- and α_{1b}-AR subtypes in HEK 293 cells,

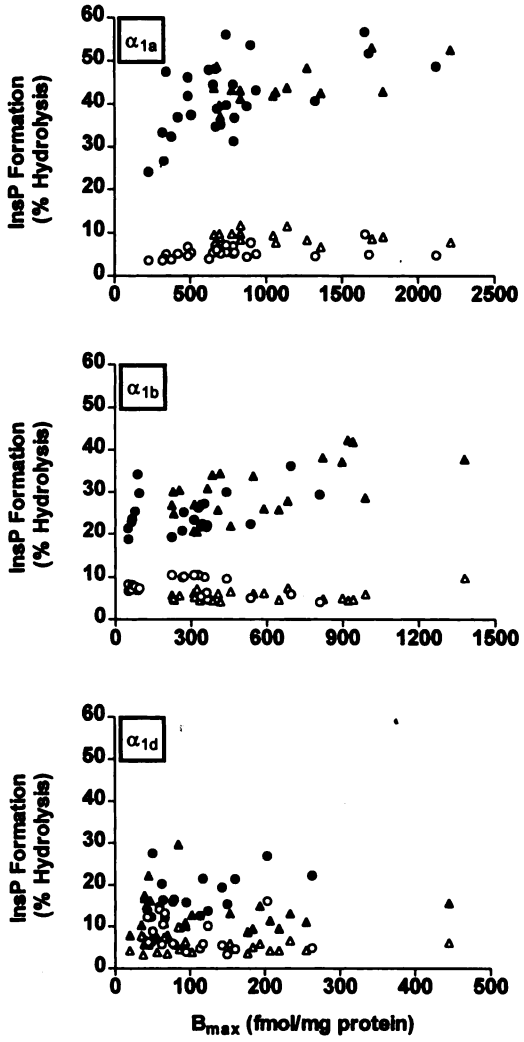

Fig. 3. Effect of density of α_1-AR subtypes on NE-stimulated InsP formation in subclones of transfected HEK 293 cells from compiled data. *Top*, α_{1a}-AR subclones no. 3 (●, ○) and no. 17 (▲, △). *Middle*, α_{1b}-AR subclones no. 9 (●, ○) and no. 20 (▲, △). *Bottom*, α_{1d}-AR subclones no. 5 (●, ○) and no. 27 (▲, △). Subclones were exposed for various time periods to 1 μg/ml ANTET or no ANTET to induce a wide range of expression levels. Duplicate plates were then incubated with (closed symbols) or without (open symbols) 100 μM NE for 1 hr, and [^{3}H]InsP formation was measured as described in Experimental Procedures. Data are expressed as percentage hydrolysis of total ^{3}H-labeled phospholipid and represent the mean of duplicate determinations. Saturation of ^{125}I-BE binding in membranes prepared from cells treated identically was analyzed by nonlinear regression to determine receptor density (B_{max}). Data are from three to five individual experiments on each subclone, each of which includes seven time points of ANTET exposure. The slopes of the regression lines for the α_{1a} (p = 0.0004) and α_{1b} (p = 0.0001) subtypes are significantly different from zero in the presence of NE. However, the slope of the regression line for the α_{1d} (p = 0.69) subtype is not significantly different from zero.

no significant increase is seen with the α_{1d}-AR subtype (the slope of the regression line through values for NE-stimulated InsP formation is not significantly different from zero).

Altered expression of α_1-AR subtypes does not affect basal or NaF-stimulated InsP formation. It was important to ensure that the relationship between α_1 AR density and NE-stimulated InsP formation is an accurate reflection of the coupling efficiency of each subtype. It is possible that phenotypic changes might have occurred during expansion and propagation of individual clonal cell lines and/or that important signaling proteins might have been affected by ANTET exposure. To control for these possibilities, we measured both basal and NaF-stimulated InsP formation in each experiment.

Fig. 4 (*top*) shows compiled data for mean basal and NaF-stimulated InsP responses in all experiments from each subclone studied. Although some variability was observed, it could not account for the differences observed in InsP responses to different α_1-AR subtypes. The α_{1a}-expressing subclones showed the largest variability in both basal (5.5 ± 0.3% to 9.3 ± 0.4%) and NaF-stimulated (11.3 ± 1.0% to 23.2 ± 0.9%) levels of InsP formation, whereas basal and NaF responses were similar in α_{1b}- and α_{1d}-expressing subclones. When data from multiple subclones expressing each subtype were averaged, there were no significant differences between basal or NaF-stimulated InsP for the subclones expressing the different subtypes (Fig. 4, *bottom*). Finally, both basal and NaF-stimulated InsP formation were relatively constant at all levels of α_1-AR expression for all three subtypes (Fig. 5). No significant correlation was observed between basal or NaF responses and receptor expression for any of the three subtypes, with linear regression resulting in slopes not significantly different from zero.

Comparison of responses over the same ranges of receptor densities. The cell lines expressing the α_{1a}-, α_{1b}-, and α_{1d}-AR subtypes showed different levels of NE-stimulated InsP formation but also different levels of receptor expression. To control for these different expression levels and to directly compare coupling efficiencies of the different subtypes, we examined responses of subclones within a limited common range of receptor expression. The α_{1a}- and α_{1b}-expressing subclones were compared over the range of 500-1000 fmol/mg of protein, whereas α_{1b}- and α_{1d}-ARs were compared over the range of 50–250 fmol/mg of protein. The data points included within each range of receptor expression (a subset of the data in Fig. 3) showed no significant differences in distribution (Table 1), allowing us to directly compare mean responses within these ranges.

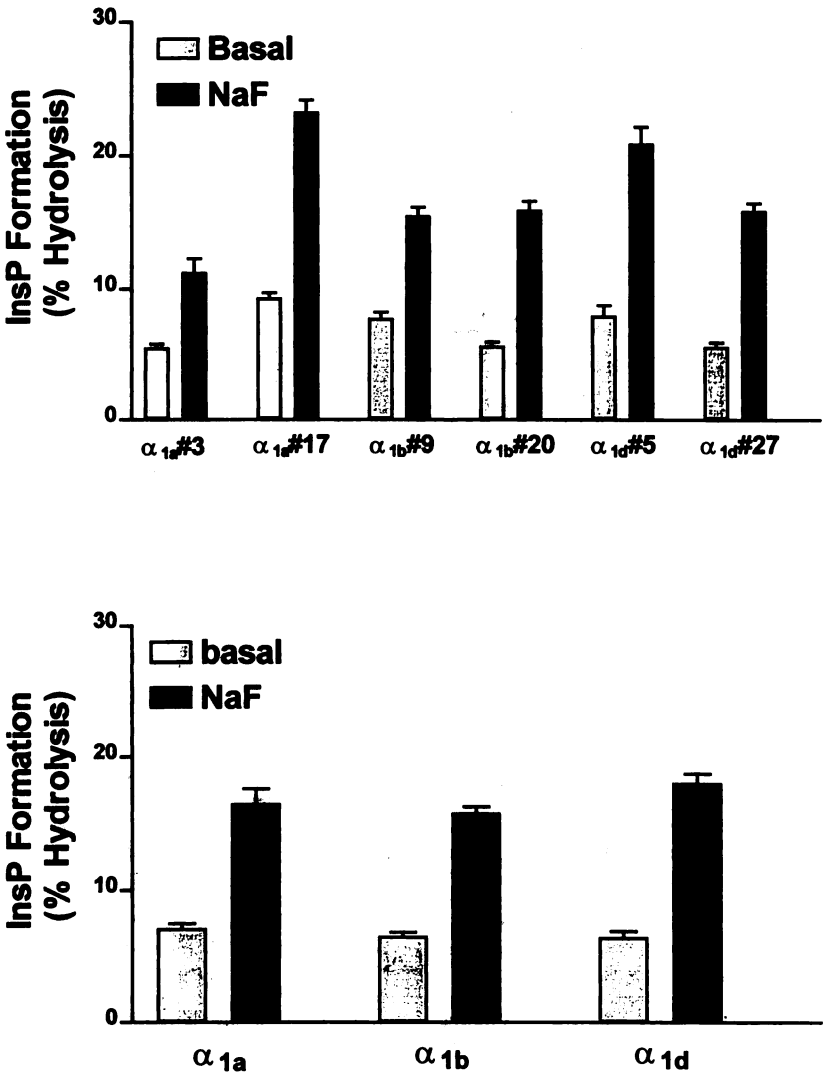

Fig. 4. Basal and NaF-stimulated InsP formation in HEK 293 subclones expressing different α_1-AR subtypes. Subclones of HEK 293 cells expressing human α_1-AR subtypes were incubated in the absence (basal) or presence of 20 mM NaF for 1 hr, and total [^{3}H]InsP formation was measured as described in Experimental Procedures. *Top*. data represent the mean ± standard error (21–28 duplicate measurements) of the percentage hydrolysis of the ^{3}H-labeled phospholipid pool for individual transfected HEK 293 subclones. *Bottom*, compiled data represent the mean ± standard error of the percentage hydrolysis of the ^{3}H-labeled phospholipid pool for transfected HEK 293 subclones expressing the same α_1-AR subtype. Data from all experiments are combined.

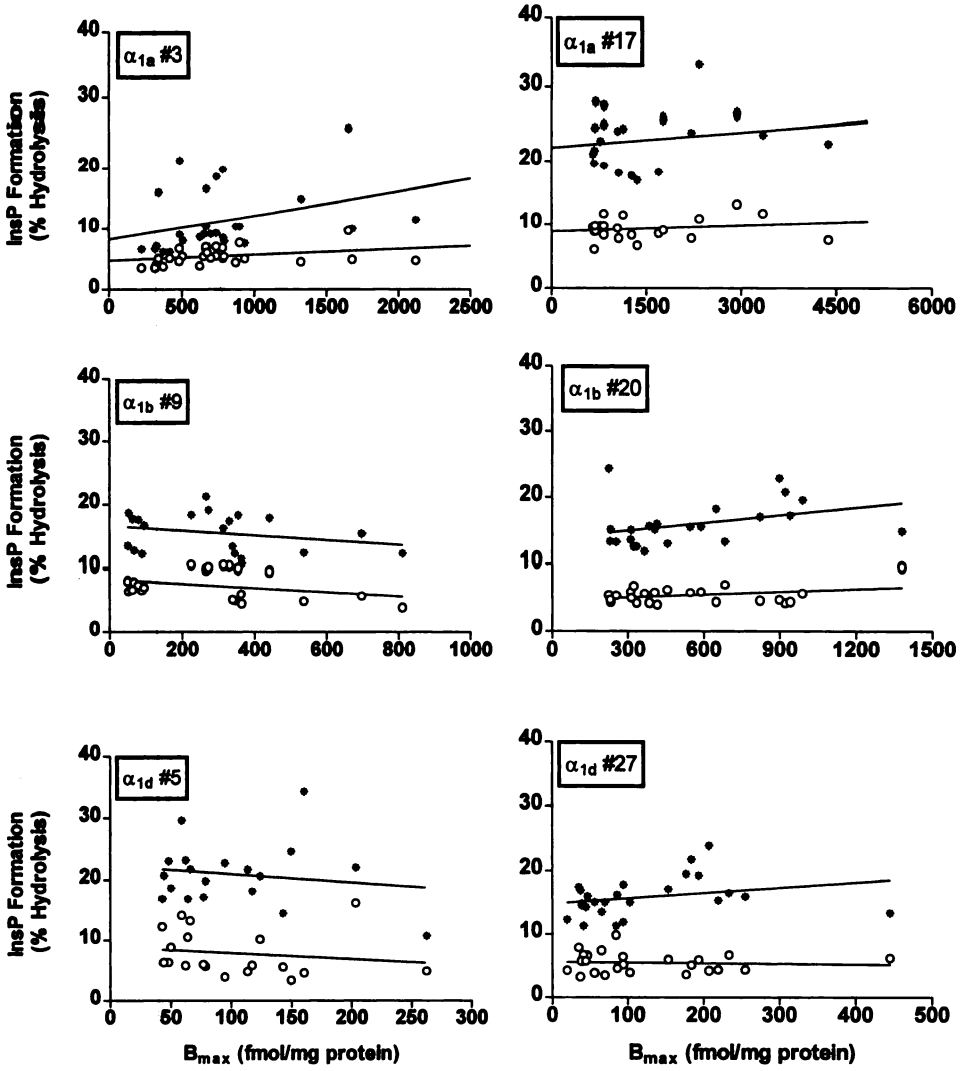

Fig. 5. Lack of correlation between α_1-AR subtype expression and basal and NaF-stimulated InsP formation. Subclones (subtype and subclone number are designated on individual graphs) were exposed to 1 μg/ml ANTET or no ANTET to induce a wide range of expression levels. Duplicate plates were incubated without (○) or with (●) 20 mM NaF for 1 hr. Data represent the mean percentage hydrolysis of the ^{3}H-labeled phospholipid pool and are compared with measurements of receptor density determined by nonlinear regression analysis of saturation of ^{125}I-BE binding in membranes. None of the slopes of the correlations were significantly different from zero when analyzed by linear regression.

TABLE 1

Distribution of α_1-AR densities (B_{max}) within 500–1000 and 50–250 fmol/mg of protein ranges of expression in HEK 293 subclones

Data are subsets of the compiled data displayed in Fig. 3. One-way analysis of variance showed no significant difference between the distribution of expression levels in α_{1a} and α_{1b} subclones within the 500–1000 ($p = 0.662$) range or between α_{1b} and α_{1d} subclones within the 50–250 range ($p = 0.665$).

α_1-AR subtype	Expression range limits	Data points within range	B_{max} (mean ± standard deviation)
	fmol/mg of protein	*n*	
α_{1a}	500–1000	24	738 ± 98
α_{1b}	500–1000	12	757 ± 160
α_{1b}	50–250	12	138 ± 84
α_{1d}	50–250	32	128 ± 63

Within the 500-1000 fmol/mg of protein expression range, α_{1a}- and α_{1b}-AR-expressing subclones showed similar levels of basal and NaF-stimulated InsP formation (Fig. 6, *top*). However, the average NE-stimulated InsP response was significantly higher for the α_{1a}-AR subtype than for the α_{1b}-AR subtype ($p < 0.05$). Within the 50–250 fmol/mg of protein expression range, α_{1b}- and α_{1d}-AR-expressing subclones also showed similar levels of basal and NaF-stimulated InsP formation (Fig. 6, bottom), but α_{1b}-AR-expressing subclones produced a significantly larger response to NE than the α_{1d}-AR-expressing cells ($p < 0.05$).

Control of α_1-AR subtype expression with an IPTG-inducible vector system in transfected SK-N-MC cells. To further compare coupling efficiencies of human α_1-AR subtypes, we studied both InsP formation and Ca^{2+} responses in human SK-N-MC cells transfected with an IPTG-inducible vector system. We have shown previously that α_{1B}-AR density can be controlled by IPTG in a concentration- and time-dependent manner in stably transfected DDT_1-MF2 cells by using this system (23). Treatment with 1 mM IPTG for 4–6 days caused a maximal increase in the density of each recombinant human α_1-AR subtype in SK-N-MC cells transfected with that subtype (data not shown). A maximal 4–6-fold increase in total α_1-AR binding sites was seen in each case (Table 2). Before induction, the densities of α_1-AR binding sites were very similar in subclones expressing all three subtypes (27–33 fmol/mg of protein) and similar to those in untransfected SK-N-MC cells (10), suggesting little constitutive activity of the vector in the absence of IPTG. Induction of receptor expression with 1 mM IPTG for 4–6 days resulted in similar induced levels in each cell line (111–190 fmol/mg of protein; Table 2). These expression levels are lower than those observed in HEK 293 cells and

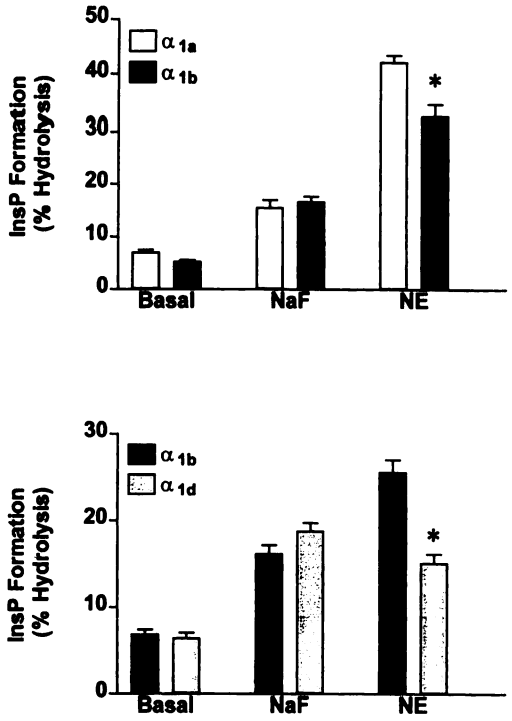

Fig. 6. Comparison of NE-stimulated InsP formation by different α_1-AR subtypes over the same range of receptor expression in transfected HEK 293 cells. *Top*, levels of basal and NaF- (20 mM) and NE- (100 μM) stimulated InsP formation in HEK cells expressing α_{1a}- and α_{1b}-AR subtypes within a range of 500-1000 fmol/mg of protein. *Bottom*, levels of basal and NaF- (20 mM) and NE- (100 μM) stimulated InsP formation in HEK cells expressing α_{1b}- and α_{1d}-AR subtypes within a range of 50–250 fmol/mg of protein. Data are subsets of data given in Fig. 3 and are presented as mean ± standard error. Table 1 shows that average receptor expression was similar for each of the subtypes within these ranges. *, $p < 0.05$, significant differences within a treatment group (Student-Newman-Keuls analysis of variance).

are more likely to reflect a physiological level of expression.

Induction of different α_1-AR subtypes in SK-N-MC cells produces different maximal effects on NE-stimulated InsP formation. Subclones of transfected SK-N-MC cells were treated with or without 1 mM IPTG for 4–6 days to determine the effect of increased expression of each human α_1-AR subtype on NE-stimulated InsP formation (Fig. 7). Increasing receptor density with IPTG caused increases in the maximal response to NE (Table 2). The greatest increase in maximal response occurred in cells expressing α_{1a}-ARs. When levels of this subtype were induced 4.8-fold over the level of endogenous receptor expression, the maximal NE-stimulated InsP response increased 7.1-fold above basal levels. In contrast, when expression of α_{1b}- and α_{1d}-ARs were increased to a similar extent (5.8- and 4.0-fold, respectively), the maximal NE-stimulated InsP responses increased only 2.9- and 2.7-fold over basal levels, respectively. The effect of increased expression of each subtype on the potency of NE in stimulating InsP formation can be seen in Fig. 7 and Table 2. Although the maximal response to NE increased with the induction of each subtype, EC_{50} values for NE did not change significantly after induction of any of the three subtypes.

Induction of different α_1-AR subtypes in SK-N-MC cells produces different maximal effects on NE-stimulated increases in $[Ca^{2+}]_i$. We also examined NE-stimulated increases in $[Ca^{2+}]_i$ in SK-N-MC cells to determine whether a similar pattern occurred. Fig. 8 shows that increasing the expression of the different α_1-AR subtypes caused effects on NE-stimulated increases in $[Ca^{2+}]_i$ in SK-N-MC cells similar to those seen with InsP formation. Increasing receptor expression through IPTG exposure caused increases in the maximal $[Ca^{2+}]_i$ response to NE (Fig. 8, *top*) with only α_{1a}- and α_{1b}-ARs. There was little effect of induction of α_{1d}-ARs on this response in SK-N-MC cells. The greatest increase in the maximal NE-stimulated increase in $[Ca^{2+}]_i$ occurred in transfected SK-N-MC cells expressing α_{1a}-ARs. The maximal NE-stimulated increase in $[Ca^{2+}]_i$ was 9.3-fold over basal levels when the receptors were induced 4.8-fold over the endogenous level of receptors. The NE-stimulated increase in $[Ca^{2+}]_i$ in induced α_{1b}-AR-transfected cells was only 2.7-fold greater than basal $[Ca^{2+}]_i$ levels despite a 5.8-fold increase in the level of receptor expression. The level of expression of the α_{1d}-AR subtype in transfected SK-N-MC cells was increased 4-fold, but the maximal increase in NE-stimulated $[Ca^{2+}]_i$ was only 2.0-fold greater than the basal $[Ca^{2+}]_i$ levels, essentially the same NE-stimulated response seen in uninduced, control cells. Again, the EC_{50} values of NE for stimulating the $[Ca^{2+}]_i$ responses remained relatively constant for all three subtypes under either control or induced levels of receptors (Fig. 8 and Table 2).

Effect of induction of α_1-AR subtypes on carbachol-stimulated InsP formation in SK-N-MC cells. To determine whether induction of α_1-AR expression had other effects on cell signaling, we compared basal and carbachol-stimulated InsP formation and $[Ca^{2+}]_i$ in transfected SK-N-MC cells before and after IPTG-induced expression of each subtype. Basal levels of InsP formation and $[Ca^{2+}]_i$ were not changed by increased expression of any of the three α_1-AR subtypes (Figs. 7 and 8). The effect of carbachol, a muscarinic receptor agonist, on $[Ca^{2+}]_i$ was unaffected by IPTG-induced increases in expression of any of the three subtypes (Fig. 9 and data not shown). In addition, induction of either the α_{1b}- or α_{1d}-AR subtypes did not significantly alter InsP responses to carbachol in these cells (data not shown). However, carbachol-stimulated InsP formation was significantly increased after induction of the α_{1a}-AR subtype (Fig. 9), although to a much lower extent than the response to NE.

The IPTG-induced increase in the InsP response to carbachol in α_{1a}-transfected cells is examined further in Fig. 9. Responses to both NE and carbachol were blocked by the α_1-selective antagonist prazosin, suggesting that the response to carbachol is mediated at least partially through α_1-ARs in these cells. In the presence of prazosin, there was no significant increase in the carbachol InsP response on induction of α_{1a}-ARs (Fig. 9), suggesting that it might be subsequent to α_1-AR activation.

Relationship between receptor density and responsiveness in transfected SK-N-MC cells. Fig. 10 shows the relationship between α_1-AR density in the transfected SK-N-MC cells and basal and maximal responses to NE. Increasing expression by 4–6-fold does not alter the basal level of InsP formation or $[Ca^{2+}]_i$ for any of the three α_1-AR subtypes (Fig. 10, *left*), showing a lack of agonist-independent constitutive activity of the induced receptors. However, similar increases in receptor expression reveal notable differences in NE-stimulated responses for the three subtypes (Fig. 10, *right*). The α_{1a}-AR-transfected cells elicit the largest re-

TABLE 2

Comparison of receptor expression levels and NE-stimulated signaling responses of the three human α_1-AR subtypes expressed in SK-N-MC cells

InsP formation is expressed as percentage of hydrolysis of the total ^{3}H-labeled phospholipid pool, and $[Ca^{2+}]_i$ is shown as the concentration (nM) measured by Fura-2 fluorescence. Basal and maximal responses were those measured in the absence and presence of 100 μM NE, respectively. Data are represented as the mean ± standard error of six to eight experiments performed in duplicate (InsP formation) or four experiments ($[Ca^{2+}]_i$ responses).

	α_{1a}		α_{1b}		α_{1d}	
	Control	Induced	Control	Induced	Control	Induced
^{125}I-BE binding						
B_{max} (fmol/mg of protein)	28.7 ± 6.3	138.8 ± 30.3	33.1 ± 7.9	190.5 ± 47.5	27.4 ± 8.2	110.9 ± 34.5
K_d (pM)	44.2 ± 6.5	48.6 ± 7.6	73.8 ± 18.5	93.9 ± 22.6	47.8 ± 6.0	67.3 ± 8.6
InsP formation (%)						
Basal	6.57 ± 0.64	6.19 ± 0.69	5.29 ± 0.77	5.58 ± 0.87	8.19 ± 1.00	9.38 ± 1.17
Maximal	20.68 ± 3.38	43.89 ± 4.77	10.50 ± 1.34	16.43 ± 1.49	15.06 ± 2.02	25.39 ± 4.91
$-\log EC_{50}$	5.64 ± 0.04	6.23 ± 0.09	5.54 ± 0.14	5.83 ± 0.14	5.75 ± 0.27	5.94 ± 0.20
$[Ca^{2+}]_i$ (nM)						
Basal	52.2 ± 6.45	50.25 ± 8.81	57.0 ± 3.2	65.8 ± 7.2	64.2 ± 7.0	57.5 ± 13.4
Maximal	221.8 ± 26.5	446.8 ± 57.0	121.0 ± 12.0	166.8 ± 13.0	174.0 ± 19.2	152.2 ± 12.1
$-\log EC_{50}$	5.94 ± 0.06	6.17 ± 0.16	5.41 ± 0.16	5.81 ± 0.30	5.55 ± 0.10	5.59 ± 0.08

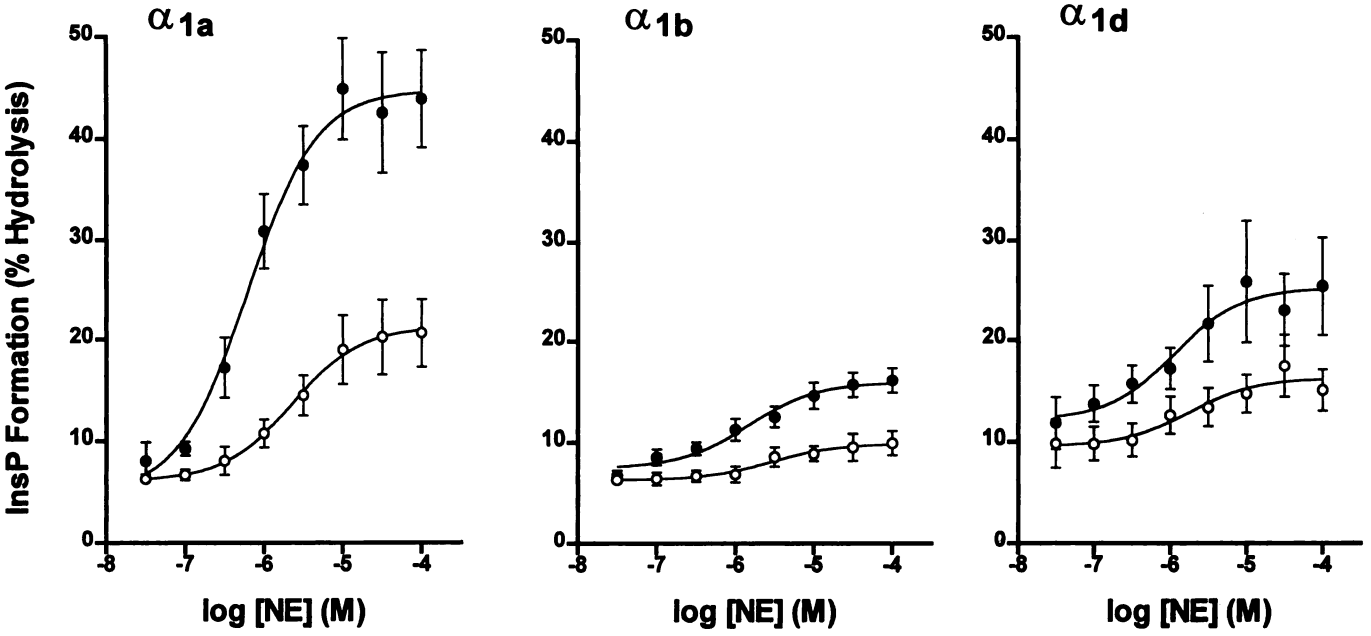

Fig. 7. Effect of IPTG induction on NE-stimulated InsP formation in subclones of SK-N-MC cells stably transfected with α_{1a}-, α_{1b}-, or α_{1d}-AR operator vectors. Subclones of cells stably transfected with human α_1-AR subtypes in IPTG-inducible vectors were labeled with 2 μCi of *myo*-[^{3}H]inositol in the absence (○) or presence (●) of 1 mM IPTG for 4–6 days. After washing, confluent cells were stimulated with increasing concentrations of NE for 1 hr, and the percentage hydrolysis of the ^{3}H-labeled phospholipid pool was determined as described in Experimental Procedures. The concentration-response *curves* were fit by nonlinear regression; *points*, mean ± standard error from six to eight experiments.

sponses for both InsP formation and increased $[Ca^{2+}]_i$, whereas α_{1d}-ARs cause very small responses in transfected SK-N-MC cells.

Discussion

We directly compared the coupling efficiencies of the three human α_1-AR subtypes in human cell lines. Cells were stably transfected with inducible or repressible expression vectors containing coding sequences for each subtype, and agonist-induced responses were examined over a wide range of receptor densities. We observed clear differences among the coupling efficiencies of the different α_1-AR subtypes. For both InsP and $[Ca^{2+}]_i$ responses, it seems that α_{1a}-ARs couple most efficiently, α_{1b}-ARs couple with an intermediate efficiency, and α_{1d}-ARs couple very poorly. Similar patterns were observed in all of the experiments despite differences in human cell type, expression vector, presence or absence of endogenous α_1-AR expression, specific subclones, range of expression levels, and type of response measured. Previous work has also raised the possibility of differences in coupling efficiencies for α_1-AR subtypes (12, 13, 15), although these have been difficult to evaluate because of differences in expression levels and potential complications due to clones from different species. This suggests that these results may have general applicability and provides the first quantitative comparison of the abilities of human α_1-AR subtypes to couple to a common signaling mechanism.

Both HEK 293 and SK-N-MC cell lines have been used

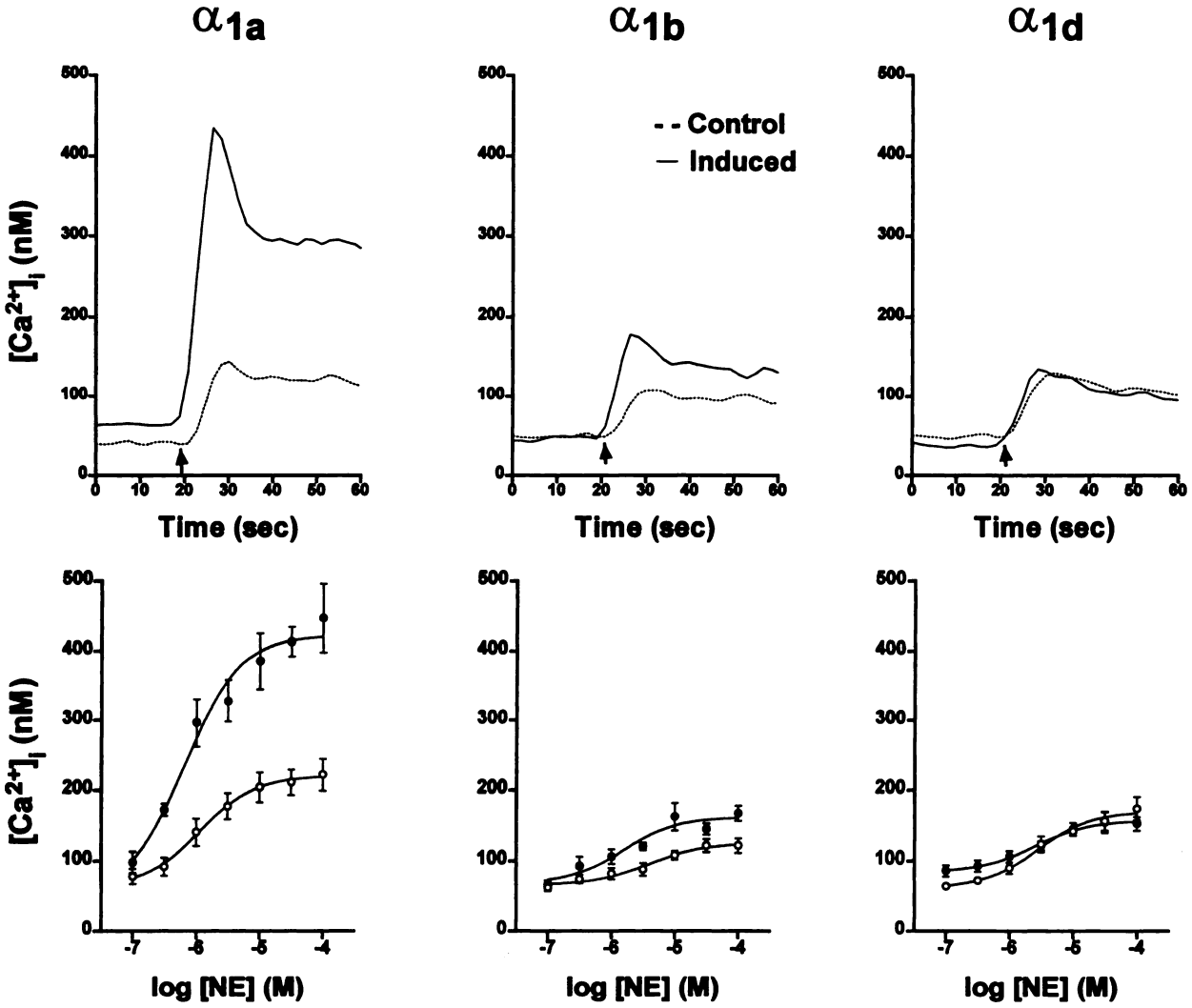

Fig. 8. Effect of IPTG induction on NE-stimulated $[Ca^{2+}]_i$ responses in subclones of SK-N-MC cells stably transfected with α_{1a}-, α_{1b}-, or α_{1d}-AR operator vectors. Subclones of cells stably transfected with human α_1-AR subtypes in IPTG-inducible vectors were incubated in the absence (*Control*) or presence (*Induced*) of 1 mM IPTG for 4–6 days. *Top*, cell suspensions loaded with Fura-2/AM were stimulated with 100 μM NE (*arrow*), and $[Ca^{2+}]_i$ measurements were determined as described in the text. Results are representative of four experiments. *Bottom*, cell suspensions were individually assayed for each indicated concentration of NE, and peak $[Ca^{2+}]_i$ was determined. The order of NE concentrations added to the cells was varied between experiments. The concentration-response *curves* were fit by nonlinear regression; *points*, mean ± standard error from four experiments.

previously to examine signaling mechanisms activated by G protein-coupled receptors, and both cell lines express the machinery necessary for coupling of α_1-ARs to InsP formation and $[Ca^{2+}]_i$. HEK 293 cells are widely used to study signal transduction pathways activated by recombinant receptors, and they express at least two G protein α subunits within the G_q family, α_q and α_{11} (22, 28). SK-N-MC cells endogenously express a wide variety of G protein-coupled receptors and are known to express α_q (29). HEK 293 cells normally express no endogenous α_1-ARs, whereas SK-N-MC cells express low levels of all three subtypes (10, 18, 21). Earlier studies (10) showed that inactivation of natively expressed α_{1B}- and α_{1D}-ARs with chloroethylclonidine did not significantly reduce NE-stimulated increases in $[Ca^{2+}]_i$ in wild-type SK-N-MC cells, despite decreasing receptor density by almost 50%. This supports the idea that similar differences in coupling efficiency occur with endogenously expressed α_1-ARs, with the α_{1a}-AR subtype coupling more efficiently than the α_{1B}- and α_{1D}-AR subtypes.

We used tetracycline-repressible (19) and IPTG-inducible (20) vectors to control α_1-AR expression. The tetracycline-repressible system resulted in higher levels of expression (≤2500 fmol/mg of protein), whereas the IPTG-inducible system gave expression at lower levels (100–200 fmol/mg of protein). Even with very high receptor expression, however, we found little evidence for increases in basal or NaF- or carbachol-stimulated responses. We did find small increases in carbachol-stimulated InsP formation, but not $[Ca^{2+}]_i$, on induction of α_{1a}-ARs (but not the other two subtypes) in SK-N-MC cells. However, this response was blocked by prazosin, suggesting that it may be due to carbachol-induced release of catecholamines. In most cases, however, non-α_1-AR-mediated responses were not altered by increasing expression of any of the three subtypes in either cell line. This indicates that the receptors do not show significant agonist-independent constitutive activity and suggests that high receptor expression does not alter other cellular signaling proteins.

Some technical difficulties were encountered with the tet-

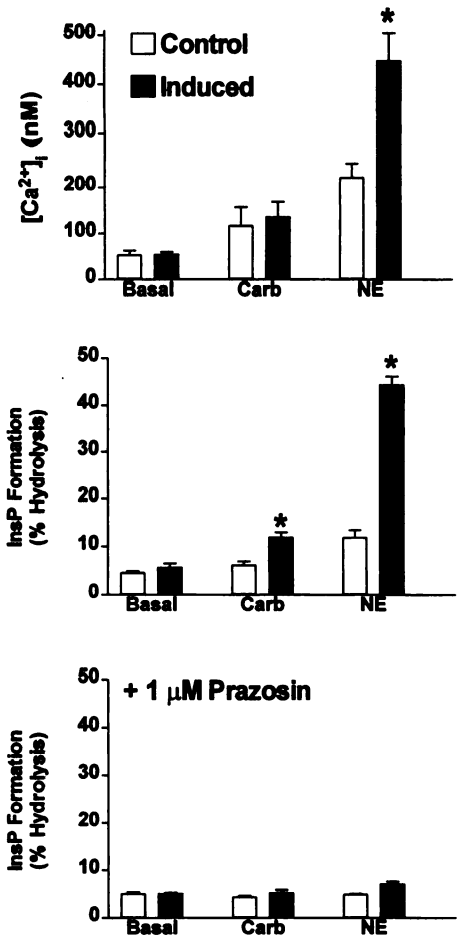

Fig. 9. Effect of IPTG induction on carbachol-stimulated responses in subclones of SK-N-MC cells stably transfected with α_{1a}-AR operator vector. *Top*, SK-N-MC cells stably transfected with the human α_{1a}-AR in an IPTG-inducible vector were incubated in the absence (*Control*) or presence (*Induced*) of 1 mM IPTG for 4–6 days as described in the legend to Fig. 7. Cells were loaded with Fura-2, and basal and carbachol- and NE-stimulated Ca^{2+} responses were determined as described in the legend to Fig. 8. *Middle*, InsP formation was determined in the absence (*Basal*) or presence of 1 mM carbachol (*Carb*) or 100 μM NE for 1 hr. *Bottom*, Similar experiments on InsP formation were performed in the presence of 1 μM prazosin to block α_1-ARs. *Bars*, mean ± standard error from four to eight experiments. *, $p < 0.05$ by Student-Newman-Keuls analysis of variance.

racycline-repressible expression system. We found positive α_1-AR expression in only 28 of 86 subclones screened after transfection and selection, and each of these subclones lost expression progressively over time, although at different rates. Shockett *et al.* (30) recently reported that the activating domain of the tTA fusion protein can be toxic to cells, which may account for the low proportion of positive subclones isolated. Modification of the vector system by placing the tTA gene under the control of its own repressible promoter was found to reduce toxicity (30) and will improve the usefulness of this system. The only major problem we encountered with the IPTG-inducible vector system seems to be some leakiness in control of the operator vector (23). This manifests itself as a basal constitutive activity of cDNA expression in the absence of IPTG, which can be substantially reduced or eliminated through screening of subclones for low basal and high inducible expression of the protein of interest.

The rank order of receptor coupling to increases in $[Ca^{2+}]_i$ in transfected SK-N-MC cells is similar to that for stimulation of InsP formation in both cell lines. Whether compared at high receptor expression in HEK 293 cells or at lower, more physiological levels of expression in SK-N-MC cells, the same order of coupling efficiency ($\alpha_{1a} > \alpha_{1b} > \alpha_{1d}$) was observed. This is especially pronounced for the α_{1a} subtype, for which even a small increase (5-fold) in receptor density in SK-N-MC cells markedly increased both responses. A similar rank order of receptor coupling to InsP and $[Ca^{2+}]_i$ responses is not unexpected because both responses are thought to be subsequent to activation of PLC. It is useful to point out, however, that these two responses were measured over very different time scales. The $[Ca^{2+}]_i$ responses were measured within seconds of the addition of agonist and presumably reflect rapid receptor activation, whereas InsP formation was influenced by events occurring over the entire 60-min exposure to NE. It is possible that other phenomena, such as desensitization (of both receptor and signaling proteins) and downstream consequences of signaling mechanisms activated (PKC activation, Ca^{2+}-dependent processes), influence InsP formation. However, the similarity of the coupling efficiencies observed for the two different responses suggests that they accurately reflect α_1-AR coupling. In fact, calcium mobilization can be viewed as a "bioassay" for formation of the active $InsP_3$ isomer, suggesting that total InsPs measured in the presence of lithium over a 1-hr exposure to agonist are a reasonable reflection of short term $InsP_3$ formation. Increased expression of each of the α_1-AR subtypes did not alter the EC_{50} value for NE in stimulating these responses, suggesting that no receptor reserve is created by increasing receptor density. Similar results were obtained with α_{1b}-ARs when InsP formation and $[Ca^{2+}]_i$ responses were examined at multiple receptor expression levels (23, 31).

The differences noted in coupling efficiency for α_1-AR subtypes are likely to reflect differences in receptor/G protein interaction, as well as subsequent interactions between G protein subunits and their effectors. Wu *et al.* (8) showed that different members of the $G_{\alpha q}$ family were differentially activated by different α_1-AR subtypes. Both $G_{\alpha q}$ and $G_{\alpha 11}$ were found to couple all three cloned α_1-AR subtypes to PLC in COS-7 cells, whereas $G_{\alpha 14}$ coupled only α_{1a}- and α_{1b}-ARs, and $G_{\alpha 16}$ coupled only the α_{1b}-AR. It is clear that discrete regions in the third intracellular loop of α_1-ARs control coupling to G proteins (32, 33), and it is possible that sequence differences among subtypes may underlie the differences in relative coupling efficiencies we observed. Because both of the cell lines that we used express $G_{\alpha q}$, which can couple all three subtypes to PLC, our results may indicate that the α_1-AR subtypes couple with different efficiencies to this G protein. However, other G protein subunits may also be expressed in these cells and play a role in coupling efficiency that has yet to be determined.

Selectivity in coupling of G protein α and $\beta\gamma$ subunits to PLCβ isoforms may also contribute to observed differences in coupling efficiency. To date, no clear differences in the ability of the $G_{\alpha q}$ class of proteins ($G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, and $G_{\alpha 16}$) to activate the multiple PLCβ isoforms have been reported (34). $G_{\alpha q}$ and $G_{\alpha 11}$ are ubiquitously expressed and cause similar activation of PLCβ_1 in transfected COS-7 cells (34), although other members of the $G_{\alpha q}$ or PLCβ family might show some selectivity. No information is yet available about the specificity of $\beta\gamma$ subunits in activating PLC isoforms. More direct

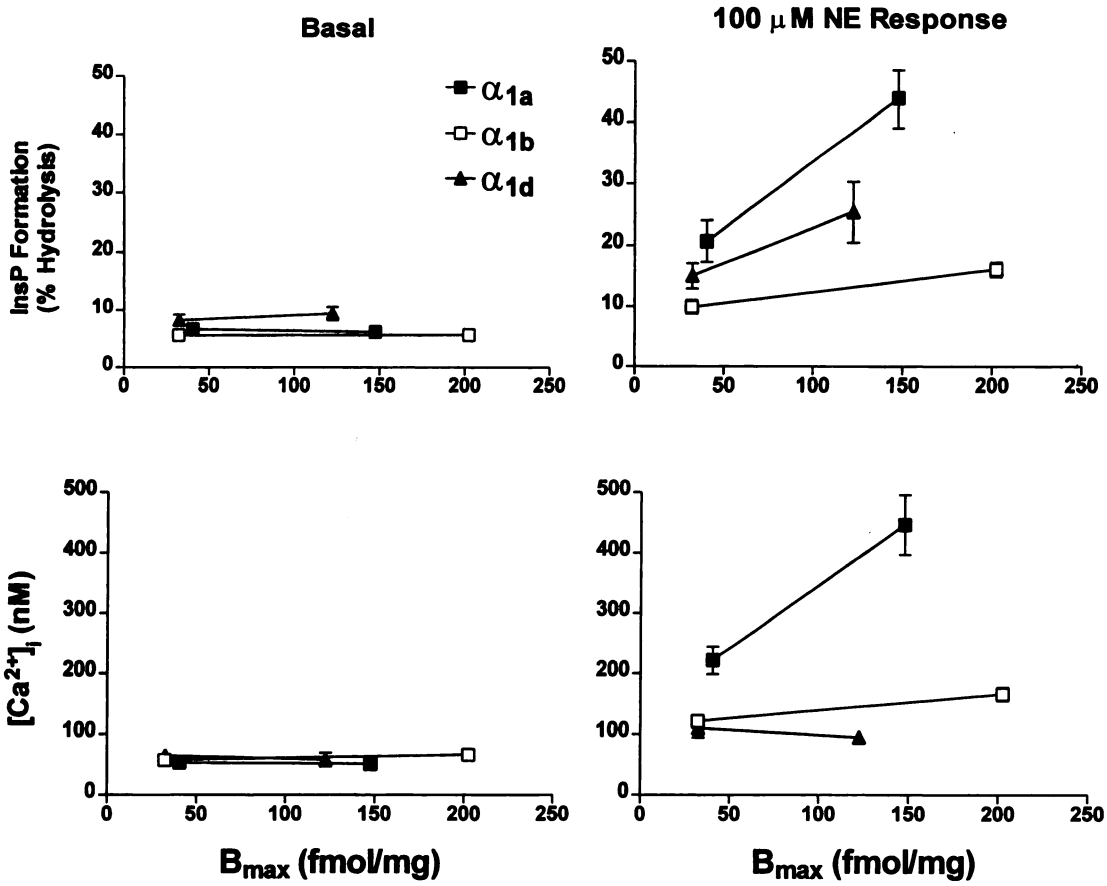

Fig. 10. Comparison of the density of α_1-AR subtypes and basal and NE-stimulated signaling responses in transfected SK-N-MC cells. *Left*, increasing α_1-AR density did not increase the basal level of InsP formation (*top*) or $[Ca^{2+}]_i$ (*bottom*). *Right*, increasing α_1-AR density increased both InsP formation (*top*) and $[Ca^{2+}]_i$ responses (*bottom*) stimulated by 100 μM NE in a subtype-dependent manner. Data are from Figs. 7 and 8; *points*, mean ± standard error from four to eight experiments.

measurement of G protein activation, such as agonist-stimulated labeling of G proteins with [α-^{32}P]GTP azidoanilide (22), would be necessary to directly address this question.

Although we found that InsP formation and mobilization of $[Ca^{2+}]_i$ are activated most effectively by α_{1a}-ARs and least effectively by α_{1d}-ARs, it is possible that these subtypes show different selectivities in activating other signal transduction mechanisms. Perez *et al.* (13) showed recently that recombinant α_{1b}- and α_{1d}-ARs couple to multiple signaling mechanisms in a G protein- and cell type-specific manner. In addition, much evidence from studies of isolated smooth muscle contraction suggests that the α_{1A} subtype may preferentially couple to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (2, 35, 36). It will be important to determine whether α_1-AR subtypes demonstrate similar differences in coupling efficiencies for activation of other second messenger responses.

In conclusion, we have shown that α_1-AR subtypes show different efficiencies in coupling to second messenger formation in two different human cell lines. The rank order of coupling efficiency was determined by comparing the ability of NE to stimulate responses at different receptor levels and was found to be $\alpha_{1a} > \alpha_{1b} > \alpha_{1d}$ for increasing both InsP formation and $[Ca^{2+}]_i$. Increasing receptor expression produced a subtype-dependent increase in the maximal response to NE without changing the EC_{50} value. These results suggest that α_1-AR subtypes differ in their abilities to activate the same intracellular signaling mechanisms. The α_{1a}-AR shows the greatest efficiency in activating InsP formation and increasing $[Ca^{2+}]_i$, whereas the α_{1d}-AR is very weak in activating both of these responses.

Acknowledgments

We thank Ms. Susanne Hollinger for excellent technical assistance.

References

1. Bylund, D. B., D. C. Eikenberg, J. P. Hieble, S. Z. Langer, R. J. Lefkowitz, K. P. Minneman, P. B. Molinoff, R. R. Ruffolo, and U. J. Trendelenberg. International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* **46**:121–136 (1994).
2. Minneman, K. P., and T. A. Esbenshade. α_1-Adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **34**:117–133 (1994).
3. Michel, M. C., B. Kenny, and D. A. Schwinn. Classification of α_1-adrenoceptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352**: 1–10 (1995).
4. Schwinn, D. A., J. W. Lomasney, W. Lorenz, P. J. Szklut, R. T. Fremeau Jr., T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and S. Cotecchia. Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.* **265**:8183–8189 (1990).
5. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *J. Biol. Chem.* **85**:7159–7163 (1988).
6. Lomasney, J. W., S. Cotecchia, W. Lorenz, W. Y. Leung, D. A. Schwinn, T. L. Yang-Feng, M. Brownstein, R. J. Lefkowitz, and M. G. Caron. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor. *J. Biol. Chem.* **266**:6365–6369 (1991).
7. Perez, D. M., M. T. Piascik, and R. M. Graham. Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor DNA. *Mol. Pharmacol.* **40**:876–883 (1991).

8. Wu, D., A. Katz, C. H. Lee, and M. I. Simon. Activation of phospholipase C by α_1-adrenergic receptors is mediated by the α subunits of G_q family. *J. Biol. Chem.* **267**:25798–25802 (1992).
9. Nakaoka, H., D. M. Perez, K. J. Baek, T. Das, A. Husain, K. Misono, M.-J. Im, and R. M. Graham, R. M. G_h: a GTP-binding protein with transglutaminase activity and receptor signalling function. *Science (Washington D. C.)* **264**:1593–1595 (1994).
10. Esbenshade, T. A., C. Han, T. J. Murphy, and K. P. Minneman. Comparison of α_1-adrenergic receptor subtypes and signal transduction in SK-N-MC and NB41A3 neuronal cell lines. *Mol. Pharmacol.* **44**:76–86 (1993).
11. Esbenshade, T. A., T. L. Theroux, and K. P. Minneman. Increased voltage-dependent calcium influx produced by α_{1b}-adrenergic receptor activation in rat medullary thyroid carcinoma 6–23 cells. *Mol. Pharmacol.* **45**:591–598 (1994).
12. Schwinn, D. A., S. O Page, J. P. Middleton, W. Lorenz, S. B. Liggett, K. Yamamoto, E. G. Lapetina, M. G. Caron, R. J. Lefkowitz, and S. Cotecchia. The α_{1c}-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.* **40**:619–626 (1991).
13. Perez, D. M., M. B. DeYoung, and R. M. Graham. Coupling of expressed α_{1b}- and α_{1d}-adrenergic receptors to multiple signalling pathways is both G protein and cell type specific. *Mol. Pharmacol.* **44**:784–795 (1993).
14. Schwinn, D. A., G. I. Johnston, S. O. Page, M. J. Mosley, K. H. Wilson, N. P. Worman, S. Campbell, M. D. Fidock, L. M. Furness, D. J. Parry-Smith, B. Peter, and D. S. Bailey. Cloning and pharmacological characterization of human α_1-adrenergic receptors: sequence corrections and direct comparison with other species homologues. *J. Pharmacol. Exp. Ther.* **272**:134–142 (1995).
15. Minneman, K. P., T. L. Theroux, S. Hollinger, C. Han, and T. A. Esbenshade. Selectivity of agonists for cloned α_1-adrenergic receptor subtypes. *Mol. Pharmacol.* **46**:929–936 (1994).
16. Hirasawa, A., K. Horie, T. Tanaka, K. Takagaki, M. Murai, J. Yano, and G. Tsujimoto. Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor. *Biochem. Biomed. Res. Commun.* **195**:902–909 (1993).
17. Ramarao, C. S., J. M. Kincade Denker, D. M. Perez, R. J. Gaivin, R. P. Rick, and R. M. Graham. Genomic organization and expression of the human α_{1B}-adrenergic receptor. *J. Biol. Chem.* **267**:21936–21945 (1992).
18. Esbenshade, T. A., A. Hirasawa, G. Tsujimoto, T. Tanaka, J. Yano, K. P. Minneman, and T. J. Murphy. Cloning of the human α_{1d}-adrenergic receptor and inducible expression of three human subtypes in SK-N-MC cells. *Mol. Pharmacol.* **47**:977–985 (1995).
19. Gossen, M., and H. Bujard. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**:5547–5551 (1992).
20. DuCoeur, L. C., D. L. Wyborski, and J. M. Short. Control of gene expression in eukaryotic cells using the lac repressor system. *Strat. Mol. Biol.* **5**:70–72 (1992).
21. Fisher, S. K., and R. E. Landon. Identification of multiple phosphoinositide-linked receptors on human SK-N-MC neuroepithelioma cells. *J. Neurochem.* **57**:1599–1608 (1991).
22. Offermans, S., T. Wieland, D. Homann, J. Sandmann, E. Bombien, K. Spicher, G. Schultz, and K. H. Jakobs. Transfected muscarinic acetylcholine receptors selectively couple to G_i-type G proteins and $G_{q/11}$. *Mol. Pharmacol.* **45**:890–898 (1994).
23. Esbenshade, T. A., X. Wang, N. G. Williams, and K. P. Minneman. Inducible expression of α_{1B}-adrenoceptors in DDT_1 MF-2 cells: comparison of receptor density and response. *Eur. J. Pharmacol.* **289**:305–310 (1995).
24. deWet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell Biol.* **7**:725–737 (1987).
25. Cowlen, M. S., and M. L. Toews. Effects of agonist and phorbol ester on adrenergic receptors of DDT_1 MF-2 cells. *J. Pharmacol. Exp. Ther.* **243**: 527–533 (1987).
26. Gossen, M., and H. Bujard. Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Res.* **21**:4411–4412 (1993).
27. Howe, J. R., B. V. Skryabin, S. M. Belcher, C. A. Serillo, and C. Schmauss. The responsiveness of a tetracycline-sensitive expression system differs in different cell lines. *J. Biol. Chem.* **270**:14168–14174 (1995).
28. Salon, J. A., J. A. Bard, C. Forray, R. L. Weinshank, and T. A. Branchek. Distribution of G protein α subunits and their coupling to cloned adrenergic α_1 receptors in commonly used transfection hosts. *Soc. Neurosci. Abstr.* **19**:938 (1993).
29. Kimura, K., B. H. White, and A. Sidhu. Coupling of human D_1 dopamine receptors to different guanine nucleotide binding proteins: evidence that D_1 dopamine receptors can couple to both G_s and G_o. *J. Biol. Chem.* **270**:14672–14678 (1995).
30. Shockett, P., M. Difilippantonio, N. Hellman, and D. G. Schatz. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **92**:6522–6526 (1995).
31. Horie, K., and G. Tsujimoto. Effect of receptor density on the receptor-effector coupling: use of cloned and stably expressed α_{1b}-adrenoceptors in CHO cells. *Eur. J. Pharmacol.* **288**:303–309 (1995).
32. Cotecchia, S., J. Ostrowski, M. A. Kjelsberg, M. G. Caron, and R. J. Lefkowitz. Discrete amino acid sequences of the α_1-adrenergic receptor determine the selectivity of coupling to phosphatidylinositol hydrolysis. *J. Biol. Chem.* **267**:1633–1639 (1992).
33. Hawes, B. E., L. M. Luttrell, S. T. Exum, and R. J. Lefkowitz. Inhibition of G protein-coupled receptor signalling by expression of cytoplasmic domains of the receptor. *J. Biol. Chem.* **269**:15776–15785 (1994).
34. Wu, D., C. H. Lee, S. G. Rhee, and M. I. Simon. Activation of phospholipase C by the α subunits of the G_q and G_{11} proteins in transfected Cos-7 cells. *J. Biol. Chem.* **267**:1811–1817 (1992).
35. Nelson, M. T., N. B. Standen, J. E. Brayden, and J. F. Worley III. Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature (Lond.)* **336**:382–385 (1988).
36. Han, C., P. W. Abel, and K. P. Minneman. α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature (Lond.)* **329**:333–335 (1987).

Send reprint requests to: Dr. Kenneth P. Minneman, Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Room 5018, Atlanta, GA 30322. E-mail: kminneman@pharm.emory.edu