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Ligand-Directed Signaling at the β_3-Adrenoceptor Produced by 3-(2-Ethylphenoxy)-1-[(1,*S*)-1,2,3,4-tetrahydronapth-1-ylamino]-2*S*-2-propanol oxalate (SR59230A) Relative to Receptor Agonists

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ABSTRACT

This study examines signaling pathways activated by the mouse β_3-adrenoceptor (AR) expressed in Chinese hamster ovary cells at high (CHOβ_3H) or low (CHOβ_3L) levels. Functional responses included extracellular acidification rate (ECAR), cAMP accumulation, and p38 mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase 1/2 (Erk1/2) phosphorylation. (−)-Isoproterenol and the β_3-AR agonist (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]1,3-benzodioxole-2,2-decarboxylate (CL316243) caused concentration-dependent increases in cAMP accumulation and ECAR in CHOβ_3H and CHOβ_3L cells. For cAMP accumulation, the β_3-AR ligand SR59230A was a partial agonist in CHOβ_3H and an antagonist in CHOβ_3L cells but for ECAR was an agonist at both expression levels. This suggested that SR59230A, which is normally regarded as an antagonist, can selectively activate pathways leading to ECAR. Examination of the pathways stimulated by (−)-isoproterenol, CL316243, and SR59230A for both ECAR and cAMP accumulation suggested that the cAMP pathway predominates in CHOβ_3H cells, whereas p38 MAPK is a major contributor to ECAR in CHOβ_3L cells and was the sole contributor to responses to SR59230A. Western blots of p38 MAPK and Erk1/2 phosphorylation confirmed that MAPKs are activated in CHOβ_3H and CHOβ_3L cells by CL316243 and SR59230A but that SR59230A has much higher efficacy. In addition, p38 MAPK phosphorylation displayed differences in drug potency and efficacy between CHOβ_3H and CHOβ_3L cells related to inhibition of the response by cAMP. Thus, CL316243 and SR59230A display reversed orders of efficacy for cAMP accumulation compared with Erk1/2 and p38 MAPK phosphorylation, providing a strong indication of ligand-directed signaling.

SR59230A was the first β_3-adrenoceptor (AR) antagonist described previously (Manara et al., 1996) and has been shown to competitively antagonize β_3-AR-mediated responses in a wide variety of tissues. However, agonist actions have been reported at the β_3-AR in guinea pig gastric fundus and duodenum (Horinouchi and Koike, 2001), rat lung (Dumas et al., 1998), rat aorta (Brahmadevara et al., 2003), and

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ABBREVIATIONS: AR adrenoceptor; CHO Chinese hamster ovary; ECAR, extracellular acidification rate; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; Erk1/2, extracellular signal-regulated protein kinase 1/2; CYP, cyanopindolol; SR59230A, 3-(2-ethylphenoxy)-1-[(1,*S*)-1,2,3,4-tetrahydronapth-1-ylamino]-2*S*-2-propanol oxalate; CL316243, (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl] 1,3-benzodioxole-2,2-decarboxylate; RWJ67657, 4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl]-3-butyn-1-ol; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PD98059, 2′-amino-3′-methoxyflavone; DDA, 2′,3′-dideoxyadenosine; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; PKA, protein kinase A; IBMX, 3-isobutyl-1-methylxanthine; BSA, bovine serum albumin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MEK, mitogen-activated protein kinase kinase; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cAMP; BRL37344, (4-(2-((2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl)phenoxy)acetic acid; CGP12177A, 4-(3-tert-butylamino-2-hydroxypropoxy)benzimidazol-2-one; SR58611A, *N*-(7-hydroxy-1,2,3,4-tetrahydronaphth-2-yl)-2-hydroxy-2-(3-chlorophenyl)ethanol; ICI118551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CHOβ_3H, β_3 adrenoceptor expressed in Chinese hamster ovary cells at high levels; CHOβ_3L, β_3 adrenoceptor expressed in Chinese hamster ovary cells at low levels.

mouse ileum (Hutchinson et al., 2005), although these responses are not associated with increases in cAMP (Hutchinson et al., 2005). Similar agonist actions have been noted in cells expressing human β_3-ARs (Strosberg and Pietri-Rouxel, 1997; Candelore et al., 1999). In CHO-K1 cells expressing mouse β_3-ARs at low levels and in 3T3-F442A cells that naturally express the receptor, SR59230A is a classic competitive antagonist for cAMP accumulation yet in the same cells causes concentration-dependent increases in extracellular acidification rate (ECAR) in the cytosensor microphysiometer and acts as an agonist with similar efficacy to the β_3-AR agonist CL316243 (Hutchinson et al., 2005). These studies suggested that the effects of SR59230A on ECAR reflect activation of signaling pathways other than cAMP rather than higher amplification of the ECAR response. A potential candidate pathway is p38 MAPK that is phosphorylated in response to β_3-AR agonists in adipocytes (Cao et al., 2001; Mizuno et al., 2002) and to SR59230A in 3T3-L1 cells (Mizuno et al., 2002). This may be the link with ECAR because p38 MAPK is known to activate the Na^+/H^+ exchanger 1 that has an important role in controlling cellular pH (Khaled et al., 2001).

It is possible that the ability of ligands such as SR59230A to block cAMP accumulation in response to β_3-AR agonists and yet activate other pathways represents an example of ligand-directed signaling. There are now a number of examples of ligand-directed signaling (Urban et al., 2007) that have been explained by the existence of multiple active conformations of receptors, termed the "conformational cafeteria" (Kenakin, 2003; Clarke, 2005). Several recent studies have described the stimulation of Erk1/2 phosphorylation by β-AR ligands usually classified as antagonists in cells expressing β_1- or β_2-ARs (Azzi et al., 2003; Baker et al., 2003; Galandrin and Bouvier, 2006). The inverse agonists ICI118551 and propranolol are antagonists for cAMP responses but agonists for Erk1/2 activation (Azzi et al., 2003; Baker et al., 2003). A recent study that examined the effects on Erk1/2 phosphorylation of a wide range of β-AR ligands after activation of β_1- or β_2-ARs found complex efficacy profiles, with compounds that acted as inverse agonists for the cAMP pathway displaying agonist, neutral antagonist, or inverse agonist properties with respect to Erk1/2 activation (Galandrin and Bouvier, 2006). There were also compounds that acted as partial agonists for cAMP accumulation but were agonists or neutral antagonists for Erk1/2 phosphorylation (Galandrin and Bouvier, 2006).

In this study, we have examined signaling pathways used by the β_3-AR in response to stimulation by the β-AR agonist (−)-isoproterenol, the selective β_3-AR agonist CL316243, and the β_3-AR ligand SR59230A in cells expressing high (CHOβ_3H) and low (CHOβ_3L) levels of the mouse β_3-AR. We find that (−)-isoproterenol and CL316243 produce equivalent responses for cAMP accumulation in both low- and high-expressing cells, whereas SR59230A is a partial agonist in high-expressing cells and a competitive antagonist of responses to CL316243 in low-expressing cells. All three ligands are agonists for ECAR in both low- and high-expressing cells. The agonist efficacy with respect to CL316243 and SR59230A is reversed for p38 MAPK and Erk1/2 phosphorylation. In both low- and high-expressing cells, SR59230A stimulates Erk1/2 phosphorylation with much higher efficacy than CL316243. Although similar in pattern to Erk1/2, p38 MAPK phosphorylation shows differences in drug potency and efficacy between CHOβ_3H and CHOβ_3L cells related to the inhibition of the response by cAMP. SR59230A therefore preferentially directs signaling to MAPK pathways.

Materials and Methods

Expression of the Mouse β_3-AR in CHO-K1 Cells. Inserts carrying the coding region of the β_{3a}-AR were created as described previously (Hutchinson et al., 2002). Plasmids were linearized with ScaI before transfection. Fifteen micrograms of each plasmid was transfected into 5×10^6 CHO-K1 cells by electroporation (270 V, 960 μF) in a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). The cells were grown for 48 h, and then stable transformants were selected in medium containing 800 μg/ml G418. Clonal cell lines were obtained by limiting dilution of mixed cell populations, and clones were expanded and analyzed for expression levels by a single point [^{125}I](−)-cyanopindolol (800 pM) binding screen. Suitable clones were grown up for a full saturation binding analysis.

Cell Culture and Treatments. CHO-K1 cells were grown as monolayers in 50:50 Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium containing 10% (v/v) fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Clonal CHO-K1 lines transfected with the β_3-AR were grown in the above media but with the addition of G418 (400 μg/ml). Cells were maintained under 5% CO_2 at 37°C, and cells were passaged every 3 to 4 days.

Radioligand Binding Assay. Cell membranes were prepared as described earlier (Hutchinson et al., 2002), and saturation binding experiments were performed (Hutchinson et al., 2002). In brief, homogenate (~10–20 μg of protein) was incubated with [^{125}I](−)-cyanopindolol (100–2000 pM) for 60 min at room temperature in the absence or presence of (−)-alprenolol (1 mM) to define nonspecific binding. Reactions were terminated by rapid filtration through GF/C filters presoaked for 30 min in 0.5% (v/v) polyethylenimine using a Packard Cell Harvester, and radioactivity was measured using a TopCount liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA). Experiments were performed in duplicate; n refers to the number of different membrane homogenate samples used.

cAMP Accumulation Studies. Cells (1×10^4/well) were grown in 96-well plates in DMEM/Ham's F-12 medium containing 0.5% (v/v) FBS for 2 days. On the day of experiment, the medium was aspirated, and appropriate drugs were diluted in stimulation buffer [1 mg/ml bovine serum albumin (BSA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 0.5 M HEPES, pH 7.4, in Hanks' balanced salt solution] added in a final volume of 100 μl. After 30 min of incubation at 37°C, the medium was removed, and 100 μl of lysis buffer [1 mg/ml BSA, 0.3% (v/v) Tween 20, 0.5 M HEPES, and 0.5 mM IBMX, pH 7.4] was added. Samples were rapidly frozen at −70°C and then thawed before assay to lyse cells before measurement of cAMP.

In experiments examining the effect of inhibitors, cells were treated with inhibitors for 30 min before stimulation with appropriate drugs. cAMP accumulation was measured using the cAMP Alphascreen method (PerkinElmer, Victoria, Australia). Samples were defrosted, and cAMP standards (10 pM to 1 μM) were prepared in detection buffer [0.4% (v/v) Hanks' balanced salt solution, 3 mM HEPES, 0.2% (v/v) Tween 20, and 0.1% (v/v) BSA, pH 7.4], and 5 μl of unknown samples or cAMP standards were transferred into a white 384-well plate. Five microliters of acceptor beads (anti-cAMP acceptor beads diluted in detection buffer) was aliquoted to each well and incubated for 30 min in the dark. Donor bead mix (15 μl of streptavidin donor beads diluted in detection buffer, 133 units/ml biotinylated cAMP) solution was added to each well, and the plate was sealed and incubated in the dark overnight. cAMP accumulation was detected using a Fusion α microplate reader (PerkinElmer). cAMP responses were expressed as a percentage of the response to

100 μM forskolin to correct for variability in cell number or viability between individual samples. The level of receptor expression influenced absolute cAMP accumulation to forskolin [42 ± 5.2 (8) pmol/well CHOβ_3H and 27 ± 1.5 (11) pmol/well CHOβ_3L]. This difference does not influence the interpretation of the data, because the comparisons are between the agonists and SR59230A, not between cells with high or low β_3-AR expression. In fact, making the additional correction for absolute forskolin response would accentuate differences between agonist and SR59230A-stimulated cAMP accumulation in high- and low-expressing cells.

Cytosensor Microphysiometer Studies. The cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) was used to measure β_3-AR-mediated increases in ECAR as described previously (Hutchinson et al., 2002, 2005). In brief, CHO-K1 cells expressing the β_3-AR were seeded into 12-mm transwell inserts (Costar; Corning Life Science, Acton, MA) at 5×10^5 cells/cup and left to adhere overnight. On the day of the experiment, cells were equilibrated for 2 h, and cumulative concentration-response curves to (−)-isoproterenol, CL316243, or SR59230A were constructed in paired sister cells with each concentration of drug exposed to cells for 14 min. Results are expressed as a percentage of the maximal response to (−)-isoproterenol. In experiments examining the effect of inhibitors, cells were treated with inhibitors for 30 min before stimulation with appropriate drugs for 30 min. All drugs were diluted in modified RPMI 1640 medium. These results are expressed as a percentage of the maximal response to (−)-isoproterenol, CL316243, or SR59230A over basal.

Western Blotting. Cells were grown in 12-well plates at 1×10^5/well in DMEM/Ham's F-12 medium containing 0.5% FBS for 2 days, and the medium was replaced (to 0% FBS) 2 h before the experiment. In time-course studies, cells were exposed to agonist for 0 to 30 min. Cells were lysed directly in each well by the addition of 40 μl of 65°C SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Cells were scraped, transferred to an Eppendorf tube on ice, and sonicated for 10 s followed by heating to 95°C for 5 min. Aliquots of the samples were separated on a 12% polyacrylamide gel and electrotransferred to a Hybond-C Extra nitrocellulose membrane (pore size, 0.45 mm; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) with a semidry electroblotter. After transfer, the membranes were allowed to soak in Tris-buffered saline for 5 min, followed by quenching of nonspecific binding (1 h at room temperature in 5% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline). Membranes were incubated overnight at 4°C with primary antibody, phospho-p38 MAPK (Thr180/Tyr184), or phospho-p44/42 MAPK (Thr202/Tyr204), diluted 1:1000. This was detected using a secondary antibody (horseradish peroxidase-linked anti-rabbit IgG diluted 1:2000) and enhanced chemiluminescence. The membranes were then stripped with 10 M urea, 50 mM sodium phosphate, 100 mM β-mercaptoethanol for 30 min at 50°C, and reprobed with the appropriate p38 MAPK or p44/42 MAPK antibody, detected with the same secondary antibody. Results are expressed as the ratio of phosphorylated to total p38 MAPK or Erk1/2 protein. All experiments were performed in duplicate with n referring to the number of independent experiments performed.

Data Analysis. All results were expressed as a mean ± S.E.M. of n experiments. Data were analyzed using nonlinear curve-fitting (Prism version 4.0; GraphPad Software Inc., San Diego, CA). Concentration-response curves were analyzed using the general equation for a sigmoid curve with a Hill slope of 1: $Y = Bottom + Top - Bottom/^{1 + 10\log EC_{50} - X}$, where Y is the response, X is the log [ligand], Bottom is the Y response value for the bottom plateau, Top is the Y response value for the top plateau, and EC_{50} is the ligand concentration corresponding to the Y value halfway between bottom and top. Statistical significance was determined using two-way analysis of variance tests or Student's t test. Probability values less than or equal to 0.05 were considered significant.

Drugs and Reagents. RWJ67657 was kindly supplied by Dr John Siekierka (Johnson & Johnson, Raritan, NJ). Drugs and reagents were purchased as follows: G418, LY294002, PP2, and PD98059 were from CalBiochem Corporation (La Jolla, CA); (−)-[^{125}I]CYP (2200 Ci/mmol) was from Perkin Elmer; (−)-alprenolol, bacitracin, IBMX, polyethylenimine, (−)-isoproterenol, CL316243, SR59230A, forskolin, 2′,3′-dideoxyadenosine (DDA), and H-89 were from Sigma Chemical Co. (St. Louis, MO); aprotinin, leupeptin, and pepstatin A were from Valeant Pharmaceuticals (Costa Mesa, CA). All cell culture media and supplements were obtained from Trace Biosciences (Castle Hill, NSW, Australia). Antibodies were obtained from Cell Signaling Technology (Danvers, MA). All other drugs and reagents were of analytical grade.

Results

Radioligand Binding Studies. Stably transfected CHO-K1 cells were examined for levels of receptor expression in saturation experiments using [^{125}I]CYP. The pK_D values and expression levels for high- (CHOβ_3H) and low (CHOβ_3L)-expressing cells were 9.0 ± 0.3 and 1150 ± 240 fmol/mg of protein and 9.5 ± 0.1 and 115 ± 6 fmol/mg of protein, respectively (n = 4). Because receptors expressed at high and low levels had similar pK_D values, this suggested that the level of expression had little or no effect on [^{125}I]CYP binding affinity. It is also known from previous studies (Hutchinson et al., 2002) that the G protein-coupling properties of β_3-ARs are retained over a wide range of expression levels and are not the result of receptor overexpression.

Effects of (−)-Isoproterenol, CL316243, and SR59230A on cAMP Accumulation in Cells Expressing β_3 Adrenoceptors at High and Low Levels. In CHO-K1 cells stably expressing high levels of the mouse β_3-AR (CHOβ_3H), (−)-isoproterenol and the selective β_3-AR agonist CL316243 had similar efficacy in promoting cAMP accumulation. SR59230A behaved as a partial agonist, with a maximum response approximately 70% of that seen with the other agonists (Fig. 1a). CL316243 displayed a 100-fold higher potency than either (−)-isoproterenol or SR59230A (Table 1). Responses in CHOβ_3L cells were uniformly lower; SR59230A did not promote cAMP accumulation (Fig. 1b) and in fact antagonized CL316243-mediated increases in cAMP levels in a concentration-dependent manner, with a pK_B value of 7.5 ± 0.3 (n = 4; Fig. 1c). In untransfected CHO-K1 cells, no effects were observed for either CL316243 or SR59230A (up to 10 μM) on cAMP accumulation (data not shown).

Effects of (−)-Isoproterenol, CL316243, and SR59230A on Extracellular Acidification Rate in Cells Expressing β_3 Adrenoceptors at High and Low Levels. Unlike cAMP accumulation, all three drugs produced similar maximal increases in ECAR in both CHOβ_3H and CHOβ_3L cells (Fig. 2, a and b). Although the V_{max} values were similar, SR59230A and (−)-isoproterenol had substantially lower potency than CL316243, and this difference was equivalent in the high- and low-expressing cells (Table 1). In untransfected CHO-K1 cells, no effects were observed for either CL316243 or SR59230A (up to 10 μM) on ECAR (data not shown), confirming that the SR59230A-stimulated ECAR response was β_3-AR-dependent. These data corroborate our previous finding that ECAR responses to SR59230A are not dependent on increases in cAMP levels in low-expressing cells (Hutchinson et al., 2005).

Effect of Inhibitors of Adenylate Cyclase and Protein Kinase A on the ECAR Response to Agonists. In cells

expressing the β_3-AR at high levels, ECAR responses to (−)-isoproterenol, CL316243, and SR59230A were significantly inhibited by the adenylate cyclase inhibitor DDA (50 μM) and by the PKA inhibitor H-89 (10 μM) (Fig. 3a). In contrast, the ECAR responses to (−)-isoproterenol, CL316243, and SR59230A in CHOβ_3L cells were unaffected by DDA or H-89 (Fig. 3b). Although the PKA inhibitor H-89 acts as an antagonist at β_1- and β_2-ARs (Penn et al., 1999), this is not the case for mouse β_3-ARs. H-89 did not reduce cAMP accumulation in either CHOβ_3H or CHOβ_3L cells (see Fig. 5) and, if anything, slightly potentiated responses as reported previously in mouse brown adipocytes (Fredriksson et al., 2001). Second, the inhibition of ECAR by H-89 in CHOβ_3H cells is mirrored by the effect of DDA (Fig. 3a). Thus, the ECAR response in high- but not low-expressing cells is associated with cAMP production and PKA activation.

Effect of Inhibitors of PI3K, Src, MEK, and p38 MAPK on the ECAR Response to Agonists. In cells expressing β_3-AR at high levels, the ECAR responses to (−)-isoproterenol, CL316243, and SR59230A were partially inhibited by the PI3K inhibitor LY294002, the Src inhibitor PP2, and the p38 MAPK inhibitor RWJ67657 (Wadsworth et al., 1999), but not the MEK inhibitor PD98059 (Fig. 4a). LY294002, PP2, and PD98059 had no effect in CHOβ_3L cells, but the ECAR responses to (−)-isoproterenol, CL316243, and SR59230A were substantially inhibited by RWJ67657 (Fig. 4b). Thus, activation of p38 MAPK formed a significant component or, in the case of SR59230A, was totally responsible for the ECAR response in the low-expressing cells.

Interaction between the cAMP and p38 MAPK Pathways. In cells expressing β_3-AR at high levels, the adenylate cyclase inhibitor DDA significantly decreased cAMP accumulation in response to (−)-isoproterenol, CL316243, and SR59230A (Fig. 5a). In contrast, treatment with the PKA inhibitor H-89 (10 μM) significantly increased cAMP accumulation in response to each of the three drugs. The p38 kinase inhibitor, RWJ67657 (10 μM) produced no significant effect on cAMP responses at high receptor expression levels. In cells expressing β_3-AR at low levels, DDA significantly decreased cAMP accumulation in response to (−)-isoproterenol and CL316243, whereas H-89 increased cAMP responses (Fig. 5b). SR59230A did not evoke a cAMP response. It is interesting that RWJ67657 significantly decreased cAMP accumulation in response to (−)-isoproterenol and CL316243,

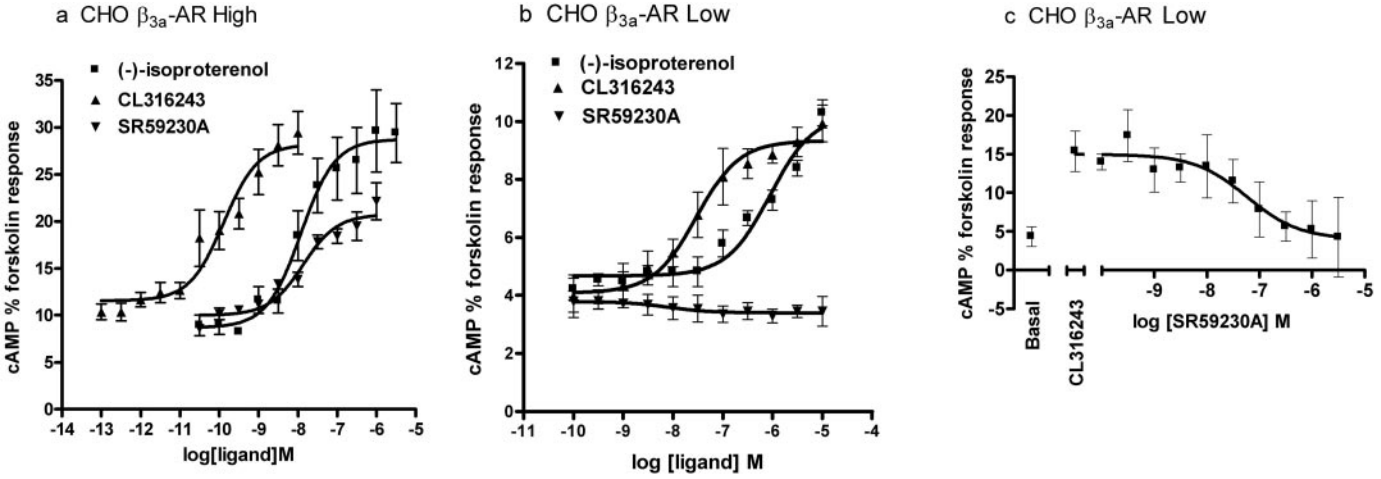

Fig. 1. Concentration-response curves for cAMP accumulation in response to (−)-isoproterenol, CL316243, or SR59230A in cells expressing β_3-AR at high (a) or low (b) levels. The results are expressed as a percentage of the forskolin (100 μM) response, and each point represents the mean ± S.E.M. (n = 4–8). (−)-Isoproterenol increased cAMP levels (CHOβ_3H max, 28.7 ± 1.4%; CHOβ_3L max, 10.3 ± 0.5%) to a similar extent to CL316243 (CHOβ_3H max, 28.2 ± 1.2%; CHOβ_3L max, 9.3 ± 0.3%). SR59230A behaved as a partial agonist relative to CL316243 in high-expressing cells (max, 20.8 ± 0.7%) but produced no significant cAMP response in CHOβ_3L cells. In c, increases in cAMP accumulation in response to CL316243 (300 nM) were antagonized by SR59230A in CHOβ_3L cells.

TABLE 1

Comparison of pEC_{50} values and V_{max} values relative to CL316243 for responses in functional bioassays and binding affinity in CHOβ_3H and CHOβ_3L cells

Binding affinities are lower than the pEC_{50} values for the strong agonists isoproterenol and CL316243 and slightly lower for the partial agonist SR59230A. The exceptions were p38 MAPK responses to SR59230A in both low- and high-expressing cells and ECAR in low-expressing cells in which the pEC_{50} value for the functional responses was lower (markedly so in the high-expressing cells) than the binding affinity (see *Discussion*).

Cell and Ligand	pEC_{50} (V_{max} relative to CL316243)				pK_i Binding
	cAMP	ECAR	p38 MAPK	Erk1/2	
CHOβ_3H					
Isoproterenol	7.8 ± 0.1 (1.02)	7.9 ± 0.3 (0.94)			4.67[a]
CL316243	9.8 ± 0.3 (1.00)	10.6 ± 0.05 (1.00)	6.1 ± 0.7 (1.00)	7.2 ± 0.4 (1.00)	5.87[a]
SR59230A	7.9 ± 0.1 (0.74)	7.8 ± 0.1 (0.89)	4.7 ± 0.2 (44.0)	7.1 ± 0.3 (2.80)	6.99[b]
CHOβ_3L					
Isoproterenol	6.2 ± 0.3 (1.11)	6.1 ± 0.2 (1.04)			
CL316243	7.6 ± 0.3 (1.00)	8.7 ± 0.1 (1.00)	7.0 ± 0.3 (1.00)	7.4 ± 0.5 (1.00)	
SR59230A	NA (0)	6.0 ± 0.1 (1.01)	6.2 ± 0.2 (3.55)	7.3 ± 0.2 (2.77)	6.93[b]

[a] Data from D. S. Hutchinson, unpublished observations.
[b] Data from Hutchinson et al. (2005).

suggesting that p38 MAPK interacts with adenylate cyclase signaling at this level of expression (Fig. 5b).

To further examine the possible interaction between adenylate cyclase signaling and p38 MAPK, we examined the effect of the cell-permeable cAMP analog 8-bromoadenosine 3′,5′-cAMP (8-Br-cAMP) on p38 MAPK phosphorylation stimulated by sorbitol and SR59230A in CHOβ_3L cells (Fig. 6). 8-Br-cAMP did not affect basal p38 MAPK phosphorylation. Sorbitol (500 mM) or SR59230A (10 μM) increased p38 MAPK phosphorylation 7- or 3-fold, respectively. The sorbitol response was significantly inhibited in the presence of 8-Br-cAMP, and the SR59230A response was completely blocked (Fig. 6).

Examination of the Effect of CL316243 and SR59230A on p38 MAPK by Western Blotting. The ECAR experiments suggested that responses involving p38 MAPK were more pronounced in cells expressing β_3-AR at low levels and greater for SR59230A than for either CL316243 or (−)-isoproterenol. We first determined time courses of p38 MAPK phosphorylation in response to concentrations of CL316243 or SR59230A that were known to give maximal ECAR responses. In low-expressing cells, exposure to both CL316243 (100 nM) or SR59230A (10 μM) increased the ratio of phospho/total p38 MAPK 4- to 6-fold, with a plateau reached after 10 to 15 min (data not shown). In high-expressing cells, on the other hand, CL316243 (1 nM) had no significant effect on the phospho/total p38 MAPK ratio and SR59230A (1 μM) caused only a 2-fold increase, again reaching a plateau at 10 to 15 min (data not shown). We next determined full concentration-response curves for CL316243 and SR59230A in both high- and low-expressing cells (Fig. 7). In CHOβ_3H cells, the maximum responses of phospho/total p38 MAPK elicited by CL316243 and SR59230A over basal were 120 and 980%, respectively. In CHOβ_3L cells, CL316243 and SR59230A caused higher p38 MAPK phosphorylation than in CHOβ_3H cells, with maximum responses of 400 and 1160% respectively. For both CL316243 and SR59230A, pEC_{50} values were higher in CHOβ_3L than in CHOβ_3H cells (Table 1). The contrast in p38 MAPK phosphorylation between high- and low-expressing cells was not due to differences in the total p38 MAPK protein levels; these were 1330 ± 210 (6) densitometric units in CHOβ_3H cells versus 1410 ± 260 (6) in CHOβ_3L cells. In both cell lines, the relative efficacy of CL316243 and SR59230A for p38 MAPK phosphorylation is a clear reversal of that seen for cAMP accumulation.

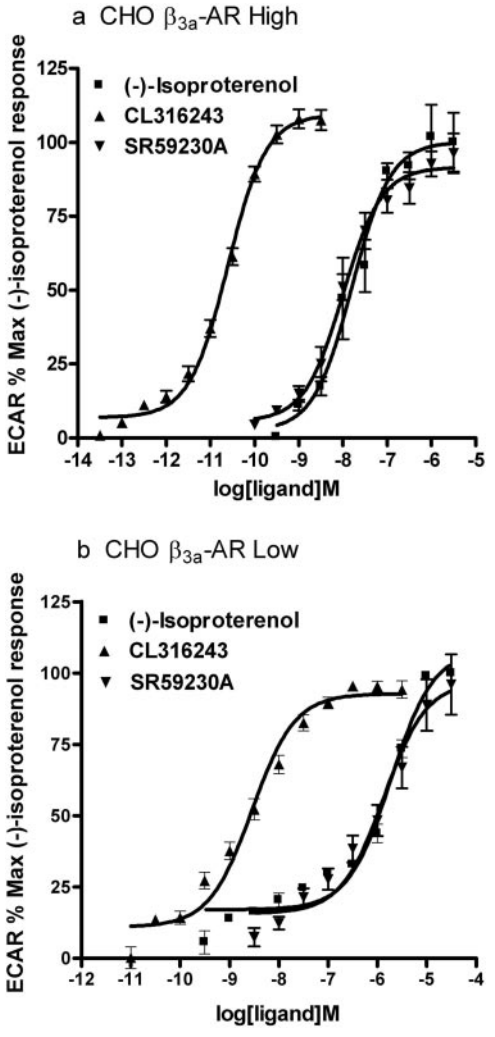

Fig. 2. Concentration-response curves for extracellular acidification rate (ECAR) in response to (−)-isoproterenol, CL316243, or SR59230A in cells expressing β_3-AR at high (a) or low (b) levels. The results are expressed as a percentage of the maximum response to CL316243. Each point represents the mean ± S.E.M. (n = 4–9). Note that CL316243, (−)-isoproterenol, and SR59230A produce equivalent responses for ECAR at both levels of receptor expression, albeit with differing potency (Table 1).

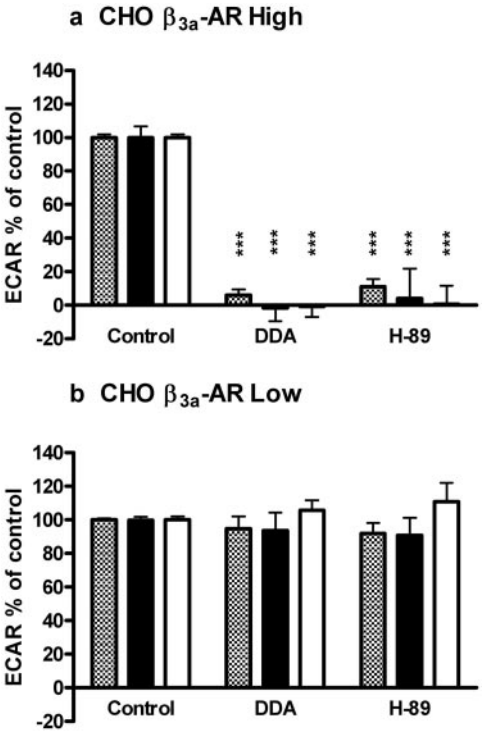

Fig. 3. The effect of inhibitors of the cAMP signaling pathway on ECAR responses to (−)-isoproterenol (▨), CL316243 (■), and SR59230A (□) in CHO-K1 cells expressing β_3-AR at high (a) and low (b) levels. The results are expressed as the percentage of increase from control induced by the agonist over basal ECAR. Each point represents the mean ± S.E.M. (n = 4–7). In CHOβ_3H cells (a), the adenylate cyclase inhibitor DDA (50 μM) substantially inhibited (***, $P < 0.001$) ECAR responses to 100 nM (−)-isoproterenol, 100 pM CL316243, and 100 nM SR59230A, as did the PKA inhibitor H-89 (10 μM), but in CHOβ_3L cells (b), the same inhibitors had little or no effect on responses to 10 μM (−)-isoproterenol, 50 nM CL316243, and 10 μM SR59230A. Concentrations of (−)-isoproterenol, CL316243, and SR59230A used for the inhibitor studies were determined from concentration-response curves to produce 80 to 90% of maximal responses.

Examination of the Effect of CL316243 and SR59230A on Erk1/2 by Western Blotting. Because recent studies have demonstrated that a number of ligands acting as inverse agonists at the β_1- and β_2-AR for cAMP accumulation display the properties of agonists, neutral antagonists, or inverse agonists for Erk1/2 activation (Azzi et al., 2003; Baker et al., 2003; Galandrin and Bouvier, 2006), we also examined the effects of CL316243 and SR59230A on Erk1/2 phosphorylation in CHO-K1 cells expressing β_3-AR at high and low levels. We found that in contrast to p38 MAPK phosphorylation, the level of Erk1/2 phosphorylation caused by activation of the β_3-AR was similar in low- and high-expressing cells, but as with p38 MAPK phosphorylation, the response to SR59230A was greater than the response to CL316243 (Fig. 8). In CHOβ_3H cells, the maximum responses expressed as phospho/total Erk1/2 ratio elicited by CL316243 and SR59230A over basal were 341 and 775%, respectively (Fig. 8 and Table 1). In CHOβ_3L cells, CL316243 and SR59230A caused similar phospho/total Erk1/2 responses to CHOβ_3H cells, with maximum responses of 384 and 886%, respectively (Fig. 8). CL316243 and SR59230A had comparable potency to each other and between the two cell lines (Table 1). The similarity between Erk1/2 phosphorylation in high- and low-expressing cells was reflected in the abundance of total Erk1/2 protein; this was 3280 ± 210 (6) densitometric units in CHOβ_3H cells versus 3110 ± 470 (6) in CHOβ_3L cells. In both cell lines, therefore, the relative efficacy of CL316243 and SR59230A for Erk1/2 phosphorylation was again a clear reversal of that seen for cAMP accumulation.

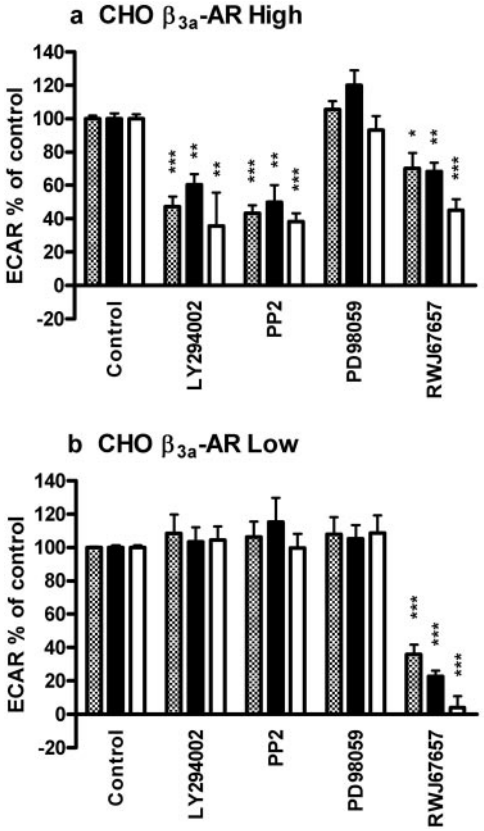

Fig. 4. The effect of inhibitors of PI3K (LY294002), Src (PP2), MEK (PD98059), and p38 MAPK (RWJ67657) on ECAR responses to (−)-isoproterenol (▨), CL316243 (■), and SR59230A (□) in CHO-K1 cells expressing β_3-AR at high (a) and low (b) levels. The results are expressed as a percentage of increase of control induced by each agonist over basal ECAR. Each point represents the mean ± S.E.M. (n = 4–6; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). In CHOβ_3H cells (a), LY294002 (10 μM), PP2 (10 μM), and RWJ67657 (10 μM) all significantly inhibited ECAR responses to 100 nM (−)-isoproterenol, 100 pM CL316243, and 100 nM SR59230A, whereas PD98059 (10 μM) had no significant effect. In CHOβ_3L cells (b), ECAR responses to 10 μM (−)-isoproterenol, 50 nM CL316243, and 10 μM SR59230A were unaffected by LY294002, PP2, or PD98059, whereas RWJ67657 caused significant inhibition, particularly of the response to SR59230A.

Discussion

CL316243 and SR59230A display reversed orders of efficacy for cAMP compared with Erk1/2 and p38 MAPK signaling in CHO-K1 cells stably expressing the mouse β_3-AR. In CHOβ_3H cells, CL316243 and (−)-isoproterenol stimulate cAMP accumulation with similar efficacy, but CL316243 is 100-fold more potent and SR59230A is a partial agonist with potency equivalent to that of (−)-isoproterenol. In CHOβ_3L cells, (−)-isoproterenol and CL316243 have reduced potency, but the cAMP response to SR59230A is lost altogether, and the compound acts as a competitive antagonist. The situation is reversed with MAPK activation in response to CL316243 and SR59230A. In both high- and low-expressing cells, SR59230A stimulates

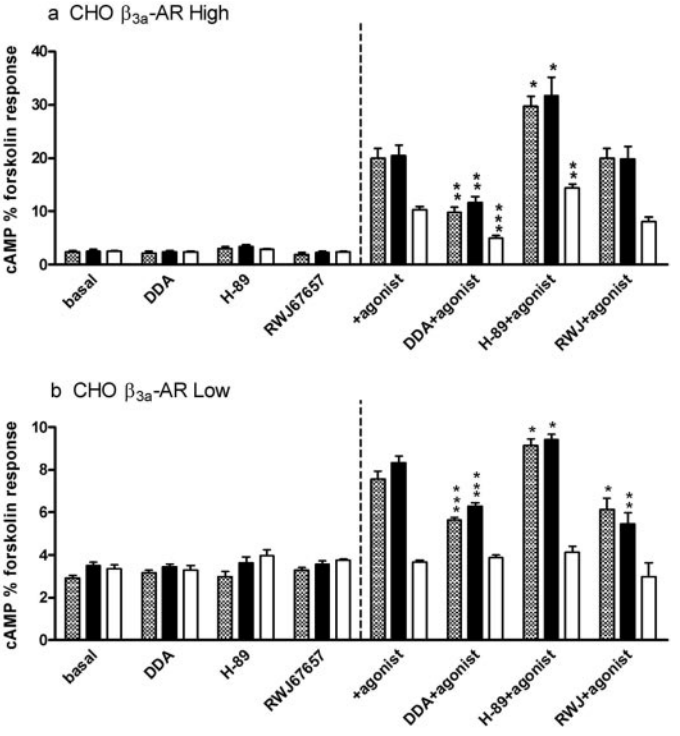

Fig. 5. The effect of inhibitors of adenylate cyclase (DDA), PKA (H-89), and p38 MAPK (RWJ67657) on cAMP accumulation responses to (−)-isoproterenol (▨), CL316243 (■), and SR59230A (□) in CHO-K1 cells expressing β_3-AR at high (a) and low (b) levels. The results are expressed as a percentage of the forskolin (100 μM) response and each point represents the mean ± S.E.M. (n = 4–6; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). In CHOβ_3H cells (a) DDA (50 μM) significantly inhibited cAMP responses to 100 nM (−)-isoproterenol, 100 pM CL316243, and 100 nM SR59230A whereas H-89 (10 μM) enhanced responses and RWJ67657 (10 μM) had no significant effect. In CHOβ_3L cells (b), DDA (50 μM) significantly decreased cAMP accumulation in response to (−)-isoproterenol and CL316243, whereas H-89 (10 μM) increased cAMP accumulation in response to (−)-isoproterenol and CL316243. RWJ67657 (10 μM) decreased cAMP accumulation in response to (−)-isoproterenol and CL316243. cAMP levels in the presence of SR59230A were unaffected by any of the inhibitors.

Erk1/2 phosphorylation with a higher efficacy than CL316243 and has an equivalent potency. This suggests that, in contrast to CL316243, the conformation of the β_3-AR recognized or induced by SR59230A is more efficiently coupled to Erk1/2 signaling than to the cAMP pathway. This interpretation requires that pathways that diverge at the receptor level mediate Erk1/2 phosphorylation and cAMP signaling. Previous studies show that β_3-AR agonists increase Erk1/2 phosphorylation in cells expressing endogenous or transfected β_3-AR (Soeder et al., 1999; Hutchinson et al., 2002). Activation of Erk1/2 involves recruitment of c-Src in adipocytes (Cao et al., 2000; Lindquist et al., 2000) and CHOβ_3-cells (Hutchinson et al., 2002). H-89, forskolin, or cholera toxin do not affect Erk1/2 phosphorylation in CHOβ_3-cells (Hutchinson et al., 2002), suggesting that the

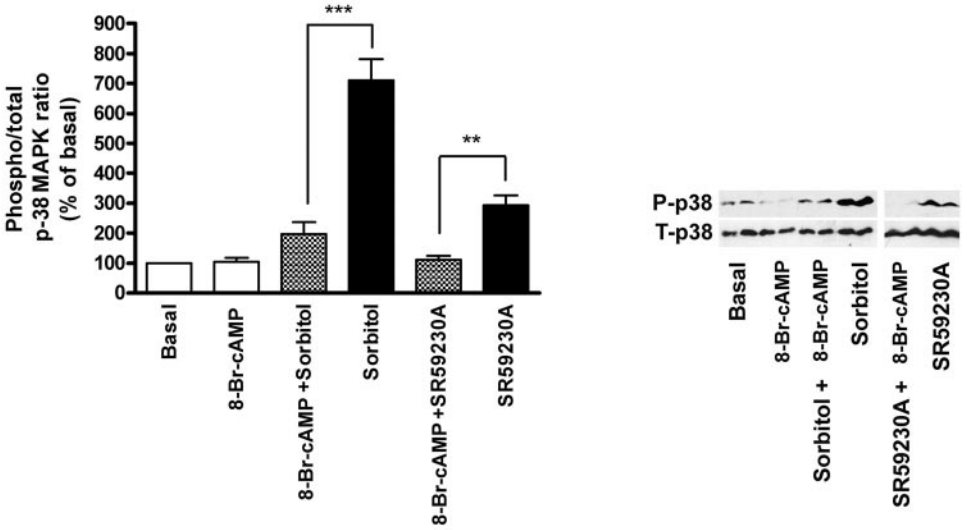

Fig. 6. Interaction between cAMP and p38 MAPK signaling in CHO-K1 cells expressing mouse β_3-AR at low levels. p38 MAPK phosphorylation was examined in response to sorbitol (500 mM) or SR59230A (10 μM) in the presence or absence of 8-Br-cAMP treatment (1 mM, 30 min). Values represent means ± S.E.M. (n = 6, performed in duplicate; ***, $P < 0.001$; **, $P < 0.01$). The immunoblot is representative of six experiments performed in duplicate. 8-Br-cAMP did not affect basal p38 MAPK phosphorylation (104 ± 14.2%). Sorbitol and SR59230A increased p38 MAPK phosphorylation (710 ± 71.2 and 294 ± 32.0%, respectively), and the responses were significantly inhibited (to 198 ± 38.7 and 111 ± 13.7%, respectively) in the presence of 8-Br-cAMP.

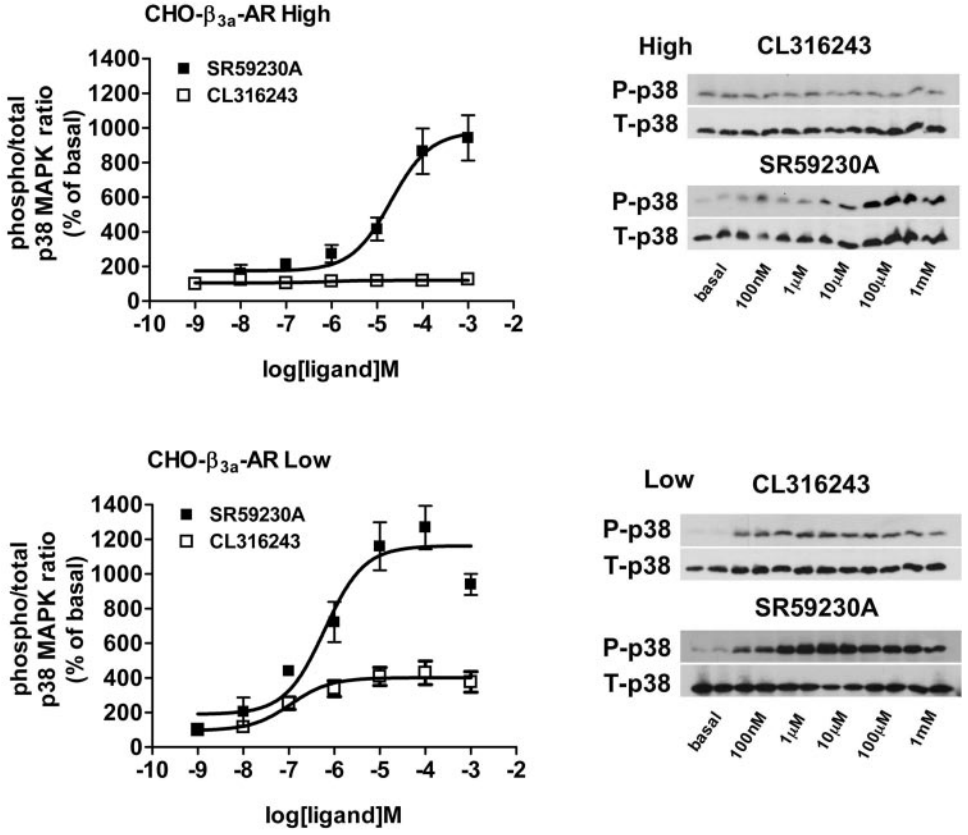

Fig. 7. Western blots of p38 MAPK phosphorylation in response to CL316243 and SR59230A in CHO-K1 cells expressing mouse β_3-AR. Concentration-response curves for p38 MAPK phosphorylation in response to 15-min exposure to CL316243 or SR59230A in cells expressing β_3-AR at high or low levels, with, at the right, representative immunoblots from six experiments performed in duplicate (P-p38 MAPK, phosphorylated p38 MAPK; T-p38 MAPK, total p38 MAPK). Each point represents the mean ± S.E.M. (n = 6, performed in duplicate). In CHOβ_3H cells, the maximum responses of phospho/total p38 MAPK elicited by CL316243 and SR59230A over basal were 120 ± 4 and 979 ± 79%, respectively. In CHOβ_3L cells, the maximum responses to CL316243 and SR59230A were 400 ± 25 and 1162 ± 69%, respectively.

$G_s\alpha$/adenylate cyclase/cAMP pathway is not involved (Gerhardt et al., 1999). Likewise, β_3-AR-mediated Erk1/2 phosphorylation is independent of G_i, because the response is insensitive to pertussis toxin, and β_{3a}-AR do not couple to G_i (Hutchinson et al., 2002; Sato et al., 2005).

p38 MAPK phosphorylation in response to β_3-AR activation occurs in 3T3-L1 adipocytes to BRL37344, CGP12177A, and SR58611A, and, to a lesser extent, SR59230A (Mizuno et al., 2002), and in these cells and primary brown adipocytes (Cao et al., 2001, 2004), the $G\alpha_s$/adenylate cyclase/cAMP pathway is necessary. We show here in CHOβ_3 cells that p38 MAPK phosphorylation to SR59230A is substantially higher than to CL316243. Although similar to Erk1/2 in terms of the relative efficacy displayed by CL316243 and SR59230A, the p38 MAPK data show differences in drug potency and efficacy between CHOβ_3L and CHOβ_3H cells. In particular, SR59230A has higher efficacy for p38 MAPK phosphorylation in CHOβ_3L than in CHOβ_3H cells and a 30-fold higher potency in CHOβ_3L cells. CL316243 produces modest p38 MAPK phosphorylation in CHOβ_3L cells but relatively little in CHOβ_3H cells. These data suggested that there is an inverse relationship between cAMP and p38 MAPK signaling in CHOβ_3 cells. Maximal p38 MAPK activation is produced by SR59230A in CHOβ_3L cells, in which it produces little (Hutchinson et al., 2005) or no (present study) cAMP accumulation. In CHOβ_3H cells, SR59230A is a partial agonist for cAMP production, and efficacy and potency for p38 MAPK activation are lower. Likewise, CL316243 produced some p38 MAPK activation in CHOβ_3L cells but very little in CHOβ_3H cells in which it powerfully activates cAMP accumulation. The relationship between receptor expression levels and

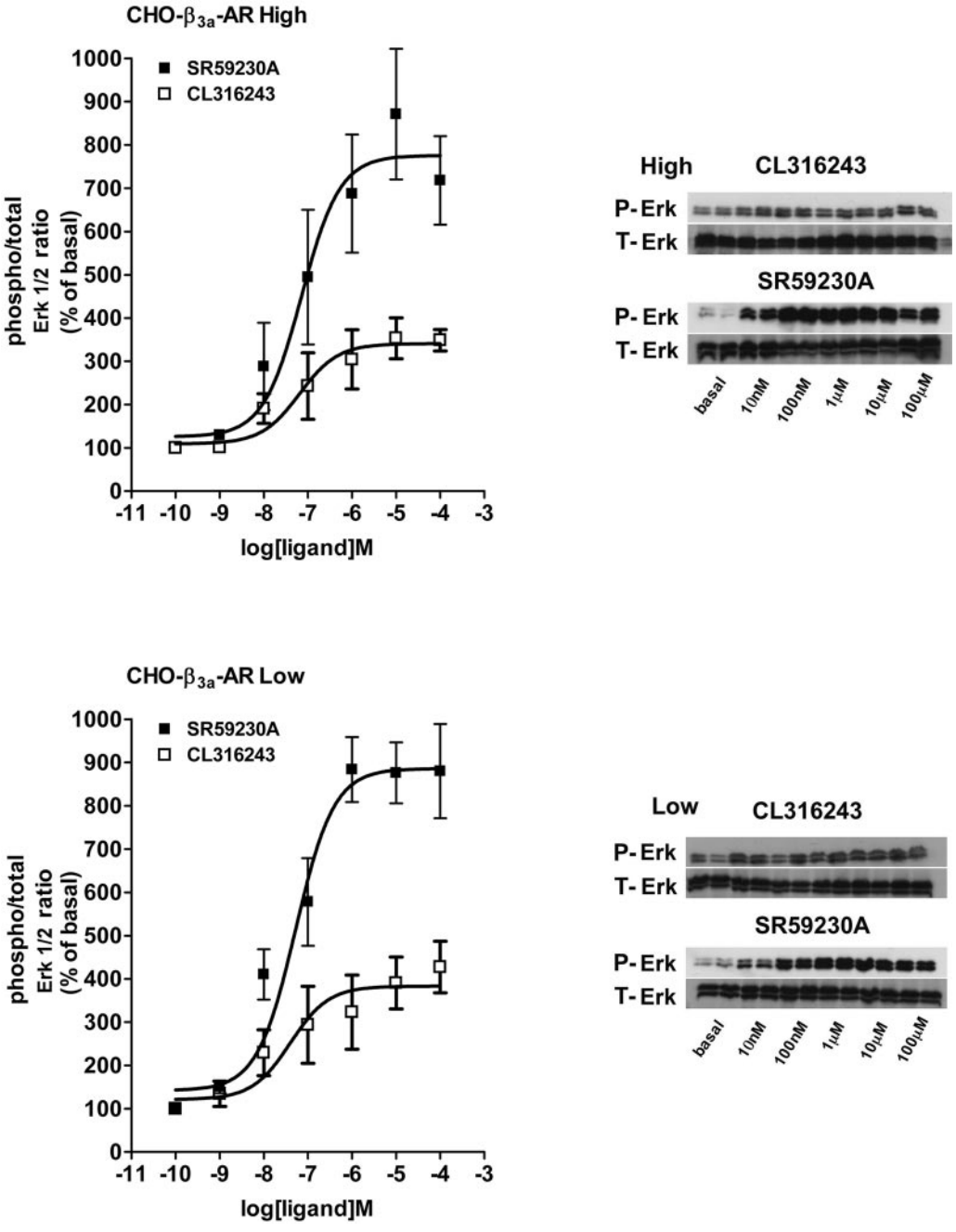

Fig. 8. Western blots of Erk1/2 phosphorylation in response to CL316243 and SR59230A in CHO-K1 cells expressing mouse β_3-AR. Concentration-response curves for Erk1/2 phosphorylation in response to 15-min exposure to CL316243 or SR59230A in cells expressing β_3-AR at high or low levels, with, at the right, representative immunoblots from six experiments performed in duplicate (P-Erk1/2, phosphorylated Erk1/2; T-Erk1/2, total Erk1/2). Each point represents the mean ± S.E.M. (n = 6, performed in duplicate). In CHOβ_3H cells, the maximum responses expressed as phospho/total Erk1/2 ratio elicited by CL316243 and SR59230A over basal were 341 ± 28 and 775 ± 68%, respectively. In CHOβ_3L cells, the maximum responses to CL316243 and SR59230A were 384 ± 36 and 886 ± 45%, respectively.

the altered p38 MAPK response therefore probably reflects the greater ability of CHOβ_3H cells to generate cAMP. This conclusion is supported by the finding that 8-Br-cAMP did not increase p38 MAPK phosphorylation but inhibited responses to both SR59230A and sorbitol.

The starting point for the present study was that in CHOβ_3L and 3T3-F442A cells, the β_3-AR ligand SR59230A is an agonist for ECAR but a competitive antagonist for cAMP responses (Hutchinson et al., 2005), suggesting that the ECAR response is mediated by pathway(s) other than cAMP. Here, we show that ECAR responses to (−)-isoproterenol, CL316243, and SR59230A in CHOβ_3L cells were unaffected by inhibitors of adenylate cyclase, PKA, PI3K, Src, or MEK but markedly inhibited by the p38 MAPK inhibitor RWJ67657. The evidence strongly suggests that p38 MAPK is a major pathway used in the ECAR response to β-AR ligands and is the sole pathway activated by SR59230A. This is consistent with the observation that pEC_{50} values for SR59230A-stimulated ECAR and p38 MAPK phosphorylation are equivalent (6.0 and 6.2, respectively; Table 1). In CHOβ_3H cells, in contrast, ECAR responses to (−)-isoproterenol, CL316243, or SR59230A were inhibited by DDA, H-89, and only partially inhibited by LY294002, PP2, or RWJ67657. Thus, the ECAR response in CHOβ_3H cells is predominantly mediated by the cAMP cascade, whereas PI3K, Src, and p38 MAPK have minor roles. This is supported by the observation that in CHOβ_3H cells, pEC_{50} values for ECAR and cAMP accumulation are similar for CL316243 (10.6 and 9.8, Table 1) and SR59230A (7.8 and 7.9, Table 1). For SR59230A, the inhibitor studies (Figs. 3 and 4) and the pEC_{50} values (Table 1) suggest a link between the cAMP and ECAR responses in CHOβ_3H cells and between p38 MAPK and ECAR in CHOβ_3L cells.

The concept of ligand-directed signaling is a topic of immense interest to pharmacologists and has been explained in terms of the ability of ligands to form distinct conformational complexes with the receptor (Kenakin, 2003; Urban et al., 2007). Recent studies have provided evidence that structurally distinct ligands differentially interact with basal state conformations of the β_2-AR to produce distinct conformational states, resulting in qualitatively different responses (Swaminath et al., 2005). Although the interaction of CL316243 and SR59230A with different conformational states of the β_3-AR may explain the differences in ability to activate cAMP accumulation and Erk1/2 phosphorylation, there is an additional layer of complexity imposed by the interaction between the cAMP and p38 MAPK signaling pathways that further accentuates the differences in pharmacological profile. Specifically, the pEC_{50} values for the four functional bioassays and binding affinities determined in previous studies are compared in Table 1. As expected, the pEC_{50} values for the recognized agonists (−)-isoproterenol and CL316243 in all four bioassays are higher than the binding affinities. The pEC_{50} values for SR59230A are much closer to binding affinities except for the p38 MAPK and ECAR (downstream of p38 MAPK) assays. There is an inverse relationship between the magnitude of the cAMP response to SR59230A and the pEC_{50} value for the p38 MAPK response. In CHOβ_3H cells in which SR59230A produced a substantial cAMP response, the pEC_{50} for p38 MAPK is lower than the pK_i value from binding, whereas in CHOβ_3L cells, in which SR59230A produced little or no cAMP, the difference is less. To have this effect, classic receptor theory suggests that generation of cAMP must be associated with a lower affinity state of the β_3-AR for SR59230A, but the mechanism involved is unclear. However, PKA phosphorylation of the receptor cannot provide an explanation, because the mouse β_{3a}-AR contains no PKA phosphorylation sites.

Several recent studies describe activation of Erk1/2 phosphorylation by drugs classified as β-AR antagonists in cells expressing β_1- or β_2-AR (Azzi et al., 2003; Baker et al., 2003). In particular, Galandrin and Bouvier (2006) demonstrated that a wide range of β-AR ligands have complex efficacy profiles for cAMP generation and Erk1/2 activation at both β_1- and β_2-ARs. Receptor-dependent activation of signaling pathways clearly depends on the array and abundance of signaling proteins present in a given cell type. We have shown that β_3-AR-mediated Erk1/2 phosphorylation displays similar properties in cells with high or low receptor abundance. In contrast, p38 MAPK responses are influenced by the level of receptor expression due to the interaction between cAMP and p38 MAPK signaling. Our demonstration of Erk1/2 and p38 MAPK signaling to SR59230A in cells expressing physiological receptor levels increases the relevance of this study to cells endogenously expressing receptors.

Given the importance of β-AR antagonists in the treatment of cardiac failure, it will be important to determine whether p38 MAPK and Erk1/2 activation in response to β-AR ligands occurs in tissues natively expressing β-ARs. p38 MAPK have important roles in cellular responses to external stress signals, such as cell growth and inflammation. Long-term overexpression of p38 MAPK in rat cardiac tissues causes cell proliferation, inflammation, and fibrosis (Tenhunen et al., 2006a), yet short-term rescue of p38 MAPK after myocardial infarction protects by decreasing left ventricular remodelling and fibrosis and enhancing angiogenesis (Tenhunen et al., 2006b). Activation of p38 MAPK by β-AR antagonists could influence a number of cardiac cell types, including myocytes, fibroblasts, and vascular endothelial cells that differentially express the three β-ARs.

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