British Journal of Pharmacology (2002) 135, 1903–1914

www.nature.com/bjp

Mouse β_{3a}- and β_{3b}-adrenoceptors expressed in Chinese hamster ovary cells display identical pharmacology but utilize distinct signalling pathways

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1 This study characterizes the mouse β_{3a}-adrenoceptor (AR) and the splice variant of the β_3-AR (β_{3b}-AR) expressed in Chinese hamster ovary cells (CHO-K1).

2 Stable clones with high (~1200), medium (~500) or low receptor expression (~100 fmol mg protein^{-1}) were determined by saturation binding with [^{125}I]-(−)-cyanopindolol. Competition binding studies showed no significant differences in affinity of β-AR ligands for either receptor.

3 Several functional responses of each receptor were measured, namely extracellular acidification rate (EAR; cytosensor microphysiometer), cyclic AMP accumulation, and Erk1/2 phosphorylation. The β_3-AR agonists BRL37344, CL316243, GR265162X, L755507, SB251023, the non-conventional partial β-AR agonist CGP12177 and the β-AR agonist (−)-isoprenaline caused concentration-dependent increases in EAR in cells expressing either splice variant. CL316243 caused concentration-dependent increases in cyclic AMP accumulation and Erk1/2 phosphorylation in cells expressing either receptor.

4 PTX treatment increased maximum EAR and cyclic AMP responses to CL316243 in cells expressing the β_{3b}-AR but not in cells expressing the β_{3a}-AR at all levels of receptor expression.

5 CL316243 increased Erk1/2 phosphorylation with pEC_{50} values and maximum responses that were not significantly different in cells expressing either splice variant. Erk1/2 phosphorylation was insensitive to PTX or H89 (PKA inhibitor) but was inhibited by LY294002 (PI3Kγ inhibitor), PP2 (c-Src inhibitor), genistein (tyrosine kinase inhibitor) and PD98059 (MEK inhibitor).

6 The adenylate cyclase activators forskolin or cholera toxin failed to increase Erk1/2 levels although both treatments markedly increased cyclic AMP accumulation in both β_{3a}- or β_{3b}-AR transfected cells.

7 These results suggest that in CHO-K1 cells, the β_{3b}-AR, can couple to both G_s and G_i to stimulate and inhibit cyclic AMP production respectively, while the β_{3a}-AR, couples solely to G_s to increase cyclic AMP levels. However, the increase in Erk1/2 phosphorylation following receptor activation is not dependent upon coupling of the receptors to G_i or the generation of cyclic AMP. *British Journal of Pharmacology* (2002) **135,** 1903–1914

Keywords: β_3-Adrenoceptor; splice variant; cytosensor microphysiometer; Erk1/2; cyclic AMP; mouse

Abbreviations: β-AR, β-adrenoceptor; BAT, brown adipose tissue; CHO-K1, Chinese hamster ovary; EAR, extracellular acidification rate: GPCR, G-protein coupled receptor; Erk1/2, extracellular-signal regulated kinase 1/2; ICYP, [^{125}I]-(−)-cyanopindolol; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase; PKA, cyclic AMP dependent protein kinase A; PTX, pertussis toxin; WAT, white adipose tissue

Introduction

Although not universal, alternative splicing of transcripts encoding GPCRs has the potential to diversify the number of receptor subtypes beyond those encoded by distinct genes. Many GPCRs have isoforms with differing C-terminal tails. These include the prostaglandin EP3 receptor (Namba *et al*., 1993; Hasegawa *et al*., 1996; Jin *et al*., 1997; Irie *et al*., 1993), α_{1A}-AR (Chang *et al*., 1998; Hirasawa *et al*., 1995), serotonin $5HT_4$ (Gerald *et al*., 1995) and $5HT_7$ receptors (Heidmann *et al*., 1997), and the somatostatin $SSTR_2$ receptor (Vanetti *et al*., 1993; Schindler *et al*., 1998). Most splice variants share similar pharmacology, but some show marked differences in signalling properties. For instance, there are four splice variants of the EP3 receptor, and each variant couples to a different set of G-proteins (Namba *et al*., 1993; Irie *et al*., 1993).

The mouse β_3-adrenoceptor (AR) gene contains two introns, both of which undergo alternate splicing (van Spronsen *et al*., 1993; Granneman & Lahners, 1995; Evans *et al*., 1999). Splicing of intron A at a novel acceptor site 100 bp upstream from the previously characterized start of exon 2 results in the production of mRNA encoding a β_3-AR variant, termed the β_{3b}-AR. This receptor has a unique C-terminal tail, with 17 amino acids (SSLLREPRHLYTCLGYP) that differ from the 13 in the known β_{3a}-AR (RFDGYEGARPFPT). There is differential expression of the two isoforms in mouse tissues, with lowest proportion of

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β_{3b}-AR transcripts in brown adipose tissue (BAT), and the highest in hypothalamus (Evans *et al*., 1999). Previous studies in mice have shown that alternate splice acceptor sites result in the generation of two β_3-AR transcripts that differ in the 3′ untranslated regions (van Spronsen *et al*., 1993: Granneman & Lahners, 1995). These transcripts have differing tissue expression in WAT and BAT, with the shorter transcript predominant in WAT, and the longer transcript in BAT. The use of primers in the 3′ untranslated region demonstrated a total of five transcripts of the mouse β_3-AR (Evans *et al*., 1999).

β_3-ARs couple to G_s to increase cyclic AMP levels but also couple to G_i proteins and c-Src, resulting in activation of the MAP kinase pathway and phosphorylation of Erk1/2. Recent studies have been performed in several cell systems including adipocyte-like cell lines that express endogenous β_3-ARs (3T3-F442A; Soeder *et al*., 1999; C3H10T1/2; Cao *et al*., 2000) and in cells transfected with the human (Gerhardt *et al*., 1999; Soeder *et al*., 1999) or mouse (Cao *et al*., 2000) β_3-AR. These studies all demonstrated β_3-AR mediated Erk1/2 phosphorylation that occurred *via* a pertussis toxin (PTX) sensitive $G_{i/o}$ protein but was independent of adenylate cyclase/cyclic AMP-dependent protein kinase A (PKA). However another study (Lindquist *et al*., 2000) using mouse brown adipocytes in primary culture showed that this $G_{i/o}$ pathway is not always necessary for β_3-AR activation of Erk1/2 phosphorylation, as in this cell type activation was mediated *via* the cyclic AMP/PKA pathway and was not affected by PTX treatment.

In this study we have characterized the pharmacological properties of the mouse β_{3a}-AR and β_{3b}-AR expressed in CHO-K1 cells. Both receptors share similar pharmacological properties with regard to affinities and potencies of β-AR ligands in radioligand binding and functional assays. Stimulation of either receptor with CL316243 increases cyclic AMP production, extracellular acidification rate (EAR) and Erk1/2 phosphorylation. Whereas the β_{3a}-AR increases cyclic AMP and extracellular acidification by signalling through G_s, the β_{3b}-AR exerts its effects by signalling through both G_s and $G_{i/o}$. Both receptors increase Erk1/2 phosphorylation *via* a mechanism independent of their effect on G_i or on cyclic AMP levels.

Methods

Expression of the mouse β_{3a}- and β_{3b}-AR in CHO-K1 cells

We generated inserts carrying the coding region of the β_{3a}- and β_{3b}-AR by RT–PCR on brown adipose tissue RNA, using high-fidelity polymerase (Expand High Fidelity PCR System, Roche). The primers used were, forward (common to β_{3a}- and β_{3b}-AR): 5′-GGAAGCTTCCCACCCCAGGC-3′, reverse (β_{3a}-AR): 5′-GAATCTAGATTCCTTGCTG-GATCTTCACGG-3′, and reverse (β_{3b}-AR): 5′-CCTTCTA-GAGAGAGCGGGACTGAGGC-3′, and included *Hind*III or *Xba*I sites for subcloning fragments into the mammalian expression vector pcDNA3.1+ (Invitrogen). The complete inserts and junctions with pcDNA3.1+ were checked by DNA sequencing on both strands (Micromon, Monash University, Australia). Plasmids were linearized with *Sca*I prior to transfection. Fifteen μg of each plasmid was transfected into 5×10^6 CHO-K1 cells by electroporation (270 V, 960 μF) in a Bio-Rad Gene Pulser II. The cells were grown for 48 h, then stable transformants were selected in medium containing 800 μg ml^{-1} G418. Clonal cell lines were obtained by limiting dilution of mixed cell populations, and were expanded and analysed by a single point [^{125}I]-(−)cyanopindolol (ICYP, 800 pM) binding screen. Suitable clones were grown further for a full saturation binding analysis.

Cell culture and treatments

CHO-K1 cells were grown as monolayers in 50 : 50 Dulbecco's modified Eagle Medium (DMEM): Ham's F-12 medium containing 10% (v v^{-1}) foetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). Clonal CHO-K1 lines transfected with the β_{3a}- or β_{3b}-AR were grown in the above media, but with the addition of G418 (400 μg ml^{-1}). All cells were maintained under 5% CO_2 at 37°C and cells passaged every 3–4 days. In experiments where cells were pre-treated with specific agents, concentration and time of treatment is indicated with the data.

Radioligand binding assay

Cells were grown to 95% confluence as a monolayer before membranes were harvested for binding studies. Cells were washed twice with HBS (10 mM HEPES, 150 mM NaCl, pH 7.4 room temperature), and scraped from flasks with lysis buffer (25 mM Tris pH 7.5 room temperature, 1 mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5 mg ml^{-1} aprotinin). Cells were homogenized with a Dounce homogeniser (approximately 10 strokes per pestle), and centrifuged at low speed ($800\times g$, 10 min) to remove cell debris. The supernatant containing membranes were retained and the pellet re-homogenized and centrifuged again. Supernatants were pooled and centrifuged ($39{,}000\times g$, 15 min, 4°C). The pellet was homogenized in binding buffer (50 mM Tris pH 7.4 room temperature, 5 mM $MgCl_2$, 1 mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5 mg ml^{-1} aprotinin) and placed on ice for use on the same day.

Experiments were performed at room temperature in a volume of 100 μl of binding buffer in 96 well microtiter plates. Homogenate ($\sim$10–20 μg protein) was incubated with ICYP (100–2000 pM) for 60 min in the absence or presence of (−)-alprenolol (1 mM) to define non-specific binding for saturation experiments. Competition experiments were performed using a range of concentrations of unlabelled drug using 500 pM ICYP. All reactions were terminated by rapid filtration through GF/C filters pre-soaked for 30 min in 0.5% polyethyleneimine using a Packard Cell Harvester. Filters were washed four times with wash buffer (50 mM Tris pH 7.4, 4°C), dried, 30 μl Microscint-O (Packard) added and radioactivity measured using a Packard Top Count. Experiments were performed in duplicate with n referring to the number of different membrane homogenate samples used.

Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., CA, U.S.A.) measures the cellular metabolic activity of isolated cells in terms of their rate of production of hydrogen ions. The basis of measurement involves the interaction of ligands with their respective receptor leading to a functional response requiring energy. Energy production and intracellular metabolic processes lead to the production and excretion of protons. The EAR is detected as a change in potential across a silicon light-addressable sensor during periods of cessation of flow of medium (McConnell *et al*., 1992). Cells were seeded into 12 mm transwell inserts (3 μm pore size) (Costar) at 5×10^5 cells per cup in media lacking FBS, and left to adhere overnight. Capsules were placed in sensor chambers in the cytosensor and maintained by a flow (100 μl min^{-1}) of modified RPMI 1640 (Molecular Devices). The flow was stopped for 40 s at the end of each 2 min pump cycle and the rate of acidification (μ volts s^{-1}) measured for 30 s during that period. Cells were superfused with media for a period of 2 h to stabilize baseline EAR before cumulative concentration–response curves to agonists were produced. Cells were exposed to each concentration of agonist for 8 min (isoprenaline) or 14 min (all other agonists used) until a stable state was achieved. All drugs were diluted in modified RPMI 1640. Baseline acidification rates were normalized using Cytosoft (Molecular Devices) prior to production of cumulative concentration–response curves. This allows comparison of results from different cell chambers in which the baseline EAR may be different due to cell density (where experiments are not performed on the same day; baseline values in cell chambers were generally similar in any given experiment performed on the same day) or other variations. The subsequent increases in EAR following agonist application to the cells is then expressed as a percentage over the increase of the normalized baseline rate. The metabolic stimulation is then normalized and scaled for each curve produced so that 0% represents the metabolic rate before addition of agonists and 100% represents the highest rate observed at the β_{3a}-AR (elicited by the highest concentration of agonist used). In the case where cells are pretreated with PTX, 100% is defined as the maximum response produced by the highest concentration of agonist in control treated cells. All *n* values represent cells grown in different flasks before plating in transwell inserts, apart from experiments where cells were treated with PTX, where paired control and treated cells were obtained from the same flask of cells.

Cyclic AMP accumulation studies

Cells (1×10^5 per well) were grown in 12-well plates in DMEM/Ham's F-12 medium containing 0.5% FBS for 2 days. On the day of the experiment, medium was replaced with one containing IBMX (1 mM) and ascorbic acid (1 mM) for 2 h before cells were exposed to drugs for 30 min unless otherwise specified. The culture medium was aspirated, 0.8 ml ice cold 75% ethanol (containing 1 mM EDTA) added, cells scraped off, and the suspension transferred to microcentrifuge tubes before being dried in a Speedvac centrifuge. The dried samples were dissolved in 50 mM Tris pH 7.4 4°C (containing 1 mM EDTA) and sonicated for a few seconds. Cyclic AMP was measured using a commercial kit (Amersham Pharmacia TRK 432). All experiments were performed in duplicate with *n* referring to number of independent experiments performed.

Erk1/2 phosphorylation

Cells (1×10^5 per well) were grown in 12-well plates in DMEM/Ham's F-12 medium containing 0.5% FBS for 2 days, and the medium replaced 2 h before experiments were commenced. Cells were exposed to agonist for 5 min unless otherwise specified. Extraction of cells and subsequent Erk1/2 phosphorylation studies were performed as previously described (Lindquist *et al*., 2000). Primary antibodies used were phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and p44/42 MAP kinase (Thr-202/Tyr-204) diluted 1 : 1000, detected using a secondary antibody (HRP linked anti-rabbit IgG) diluted 1 : 2000 and detected with the ECL kit (Amersham). Results are expressed as the ratio between phosphorylated and total Erk protein (α-p-Erk/α-Erk), with the ratio normalized in each experiment to that of control samples. All experiments were performed in duplicate with *n* referring to the number of independent experiments performed.

Data analysis

All results are expressed as mean ± s.e.mean of *n*. Data was analysed using non-linear curve fitting (GraphPad PRISM version 2.0) to obtain pEC_{50} values (cytosensor microphysi-

Table 1 Binding affinities for β-AR ligands at the β_{3a}- or β_{3b}-AR. Values are mean pK_i values ± s.e.mean with *n* values in brackets

Competitor	pK_i β_{3a}-AR	pK_i β_{3b}-AR
β_3-AR selective agonists		
BRL37344	6.02 ± 0.03 (3)	5.83 ± 0.22 (3)
CL316243	5.87 ± 0.39 (3)	5.42 ± 0.12 (3)
CGP12177A	6.54 ± 0.05 (3)	6.45 ± 0.07 (3)
β_3-AR selective antagonists		
SR59230A	7.32 ± 0.06 (5)	7.20 ± 0.08 (5)
	6.88 ± 0.08 (3)*	6.87 ± 0.10 (3)*
	6.57 ± 0.26 (3)#	6.51 ± 0.27 (3)#
β-AR nonselective agonists		
(−)-Noradrenaline	4.89 ± 0.40 (3)	4.71 ± 0.09 (3)
(−)-Isoprenaline	4.67 ± 0.17 (3)	4.65 ± 0.09 (3)
β-AR nonselective antagonists		
(−)-Propranolol	6.29 ± 0.08 (6)	5.98 ± 0.14 (6)
	6.16 ± 0.11 (3)*	6.42 ± 0.08 (3)*
	6.72 ± 0.12 (3)#	6.63 ± 0.16 (3)#
(−)-Tertatolol	6.62 ± 0.15 (3)	6.66 ± 0.13 (3)
Bupranolol	6.53 ± 0.13 (3)	6.48 ± 0.09 (3)
Labetolol	5.25 ± 0.05 (3)	5.13 ± 0.06 (3)
(−)-Alprenolol	6.54 ± 0.03 (4)	6.47 ± 0.05 (4)
β_1-AR selective antagonist		
CGP20712A	4.91 ± 0.04 (4)	4.90 ± 0.06 (4)
	4.83 ± 0.17 (3)*	5.31 ± 0.12 (3)*
	5.14 ± 0.25 (3)#	5.00 ± 0.13 (3)#
β_2-AR selective antagonist		
ICI118551	4.69 ± 0.12 (3)	4.68 ± 0.14 (3)
	5.17 ± 0.03 (3)*	5.29 ± 0.06 (3)*
	5.63 ± 0.16 (3)#	5.75 ± 0.12 (3)#

All values were determined in high expressing clones unless marked otherwise (*denotes medium and #denotes low expressing clones).

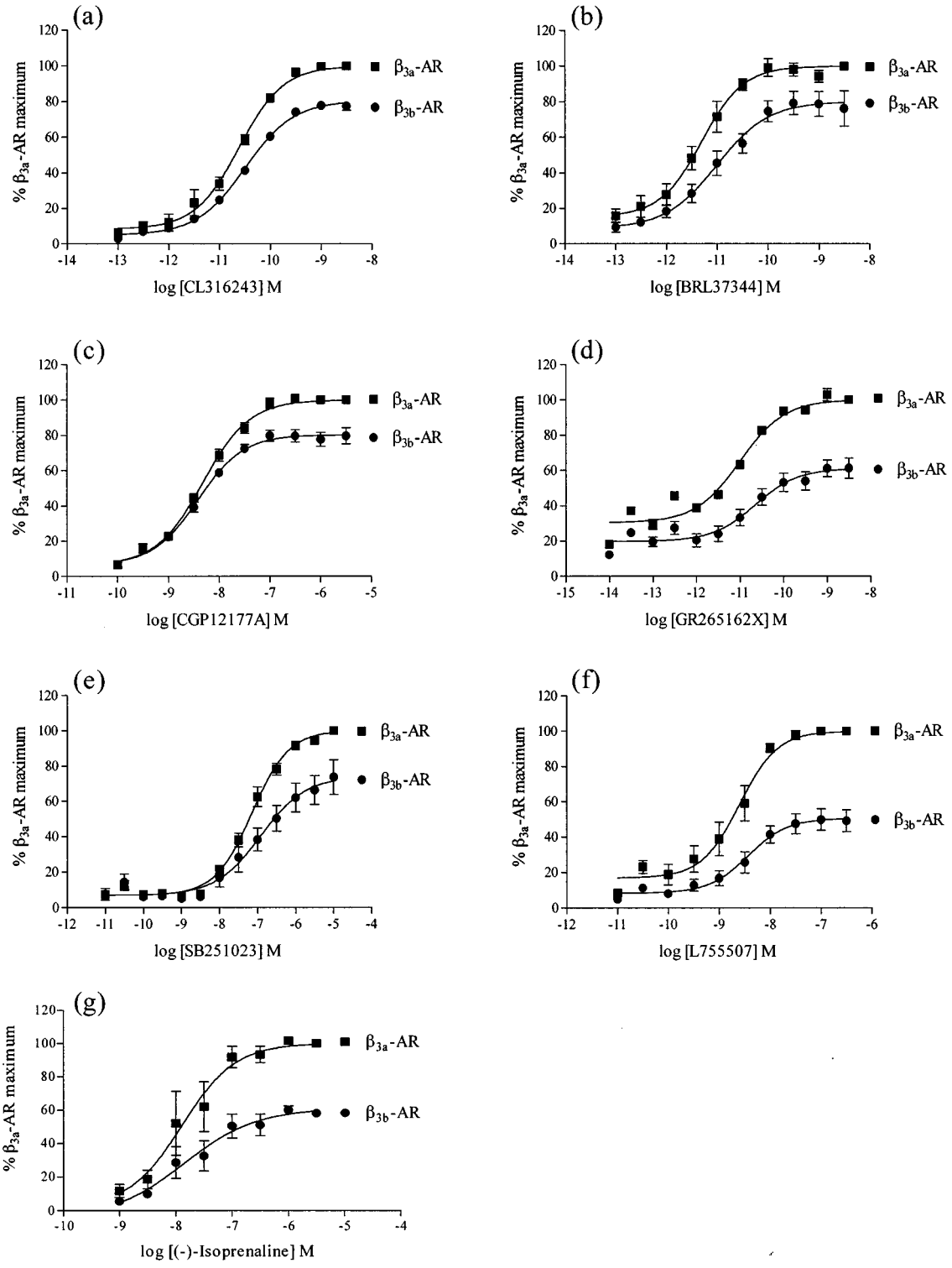

Figure 1 Concentration-response curves for CL316243 (a), BRL37344 (b), CGP12177A (c), GR265162X (d), SB251023 (e), L755507 (f) and (−)-isoprenaline (g) increasing EARs in the cytosensor microphysiometer in high expressing cells. Values represent means ± s.e.mean obtained from 3–6 experiments. Responses from CHO-K1 cells expressing β_{3b}-AR are presented as a percentage of the maximal response to the same agonist in cells expressing the β_{3a}-AR. Note the difference in x-axis for graphs.

ometer, cyclic AMP and Erk1/2 experiments) or pK_i values (competition experiments), or using a one-site fit to obtain K_D and B_{max} values (saturation experiments). Statistical significance was determined using two-way ANOVA tests or Student's *t*-test. Probability values less than or equal to 0.05 were considered significant.

Drugs and reagents

The following drugs were gifts: CL316243 (Dr T. Nash, Wyeth-Ayerst), SR59230A (Dr L. Manara, Sanofi-Midi), GR265162X (Dr Conrad Cowan, Glaxo-Wellcome), SB251023 (Dr Jon Arch, SmithKline Beecham), L755507 (Dr Mike Fisher, Merck), labetalol (Prof B. Jarrott, Monash University, Australia). Drugs and reagents were purchased as follows: BRL37344 (Tocris Cookson Ltd, Bristol, U.K.); G418, H89, PP2 (CalBiochem Corp, La Jolla, CA, U.S.A.); (−)-[^{125}I]-cyanopindolol (2200 Ci mmol^{-1}, NEN Life Science Products, Boston, MA, U.S.A.); ICI118551 (Imperial Chemical Industries, Wilmslow, Cheshire, UK); CGP12177A (Research Biochemicals Inc., MA, U.S.A.); CGP20712A (Ciba-Geigy AG, Australia); PTX (Gibco–BRL, Life Technologies, Gaithersburg, MD, U.S.A.); (−)-alprenolol, bacitracin, genistein, IBMX, (−)-isoprenaline, LY294002, (−)-norepinephrine, PD98059, polyethyleneimine, (−)-propranolol, (Sigma Chemical Company, St. Louis, MO, U.S.A.); aprotinin, leupeptin, pepstatin A (ICN, Costa Mesa, CA, U.S.A.); tertatolol (Servier Laboratories, Neuilly Sur Seine, France); bupranolol (Schwartz Pharma AG, Manheim, Germany). All cell culture medium and supplements were obtained from Trace Biosciences (Castle Hill, NSW, Australia). Antibodies were obtained from Cell Signalling Technology (Beverly, MA, U.S.A.). All other drugs and reagents were of analytical grade.

Results

Radioligand binding studies

Stably transfected cells were chosen with three levels of expression as determined with saturation radioligand binding studies: high (β_{3a}-AR B_{max} 1118±48 fmol mg protein^{-1}, K_D 921±82 pM, $n=3$; β_{3b}-AR B_{max} 1243±53 fmol mg protein^{-1}, K_D 1321±103 pM, $n=3$ (two-way ANOVA n.s.)), medium (β_{3a}-AR B_{max} 587±97 fmol mg protein^{-1}, K_D 898±38 pM, $n=3$; β_{3b}-AR B_{max} 457±67 fmol mg protein^{-1}, K_D 853±24 pM, $n=3$ (two-way ANOVA ***$P<0.001$)), and low (β_{3a}-AR B_{max} 115±6 fmol mg protein^{-1}, K_D 344±48 pM, $n=3$; β_{3b}-AR B_{max} 101±5 fmol mg protein^{-1}, K_D 372±47 pM, $n=3$ (two-way ANOVA n.s.)) levels of expression. Non-specific binding represented ~8%, ~10% or ~40% of the total binding measured in high, medium and low expressing clones respectively. The binding affinities (pK_i) of β-AR ligands measured are shown in Table 1. The affinity values for all ligands examined were not statistically different between receptors (Student's *t*-test).

Cytosensor microphysiometer studies

Exposure of CHO-K1 cells expressing high levels of the β_{3a}- or β_{3b}-AR to all β-AR ligands examined resulted in a concentration-dependent increase in the EAR relative to baseline acidification rates. Concentration-response curves to the non-selective β-AR agonist (−)-isoprenaline, β_3-AR agonists (including the rodent (CL316243, BRL37344) and human (L755507) selective agonists), the β_1-/β_2-AR antagonist/β_3-AR agonist CGP12177A, and to two new β_3-AR agonists with reputedly high selectivity for the human β_3-AR (GR265162X, SB251023) were measured (Figure 1). In all experiments performed, pEC_{50} values were similar for all agonists at both receptors when expressed at high levels, however maximal responses produced at the β_{3b}-AR were significantly less than those at the β_{3a}-AR as shown in Table 2 (two-way ANOVA ***$P<0.001$). The rank order of potency at both receptors was: BRL37344, CL316243, GR265162X > CGP12177A, L755507 > SB251023, (−)-isoprenaline. Exposure of untransfected CHO-K1 cells to CL316243 (up to 10 μM) did not increase EAR (% increase of baseline acidification rate (defined as 100%): 10 μM CL316243 CHO-K1 cells, 102%; 10 nM CL316243 β_{3a}-AR (high expressing cells), 218%). Additionally, when CL316243 concentration-response curves were produced in cells with all three expression levels in the same experiment, potency and efficacy of CL316243 was reduced when expression levels were reduced (data not shown). PTX treatment (100 ng ml^{-1} 16 h) increased maximal responses to CL316243 in cells expressing the β_{3b}-AR (two-way ANOVA ***$P<0.001$) but had no effect on responses in cells expressing the β_{3a}-AR (two-way ANOVA n.s.) at all levels of cell expression examined (Figure 2; Table 3).

Cyclic AMP accumulation studies

As seen with the cytosensor microphysiometer, cyclic AMP accumulation studies showed that in cells with high levels of receptor expression, responses produced by CL316243 at the β_{3b}-AR were significantly decreased in comparison to those produced in cells expressing the β_{3a}-AR (β_{3a}-AR maximal response 100%;

Table 2 Agonist potencies at the mouse β_{3a}- and β_{3b}-AR expressed at high levels in CHO-K1 cells assessed using the cytosensor microphysiometer

Agonist	*pEC_{50} β_{3a}-AR*	*pEC_{50} β_{3b}-AR*	*β_{3b}-AR max response % β_{3a}-AR max*	n
CL316243	10.62±0.05	10.50±0.04	80.5±1.7***	5–6
BRL37344	11.30±0.09	11.01±0.15	80.0±4.2***	4
CGP12177A	8.32±0.05	8.42±0.07	80.3±1.7***	6
GR265162X	11.00±0.08	10.65±0.18	61.1±3.6***	4
SB251023	7.14±0.04	6.91±0.20	74.0±6.8***	4
L755507	8.60±0.08	8.43±0.15	50.6±3.4***	3–4
(−)-Isoprenaline	7.88±0.25	7.85±0.43	60.9±7.3***	4

Agonist potency values are mean pEC_{50} values±s.e.mean for *n* experiments. Maximal responses in CHO-K1 cells expressing β_{3b}-AR are expressed as percentage of response produced in CHO-K1 cells expressing β_{3a}-AR, defined as 100%. ***Indicates $P<0.001$ determined by two-way ANOVA of concentration–reponse curves produced in β_{3a}- or β_{3b}-AR cells.

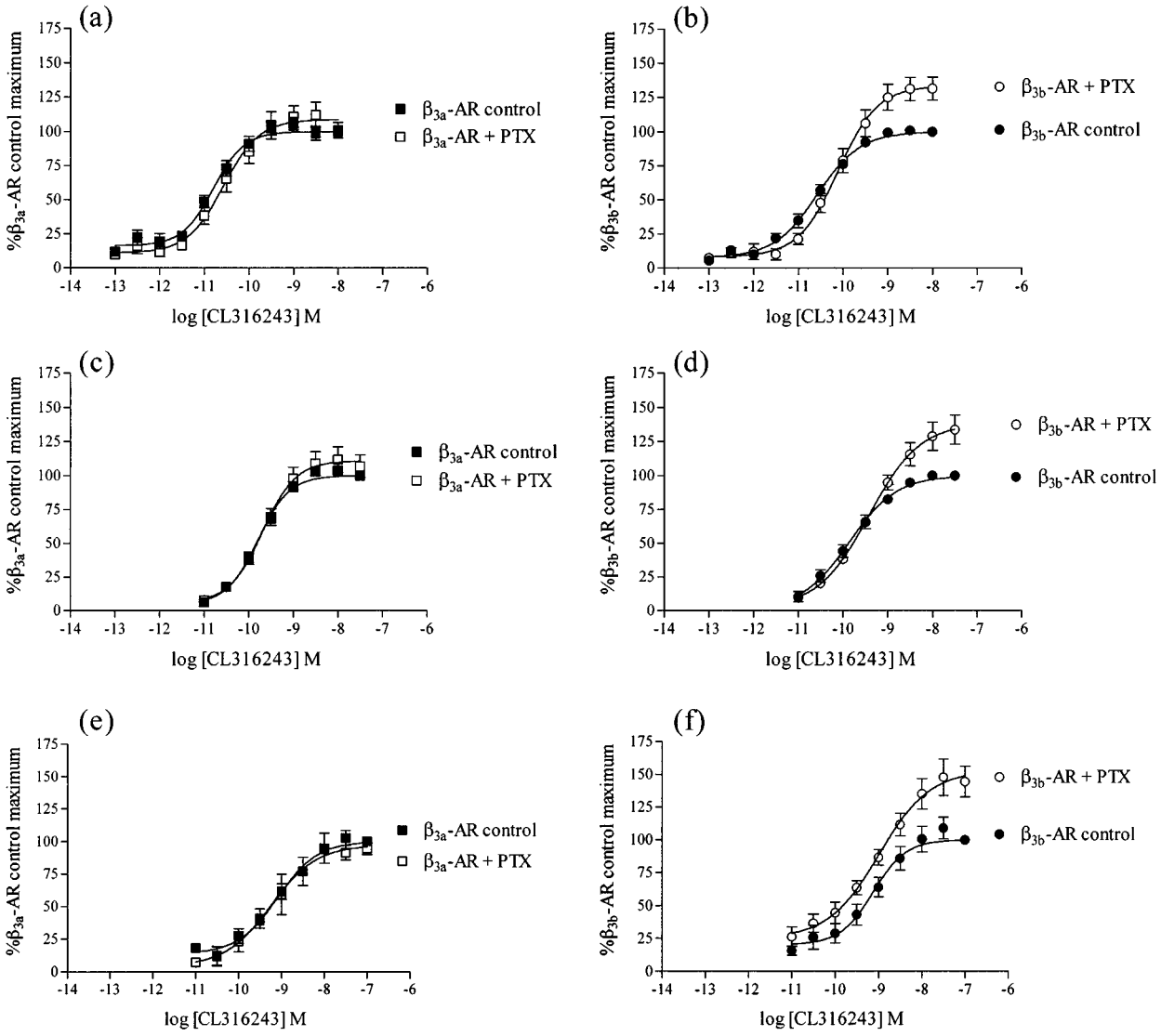

Figure 2 Effect of PTX (100 ng ml^{-1} 16 h) treatment on CL316243 extracellular acidification responses measured using the cytosensor microphysiometer in cells expressing either the β_{3a}-AR (at high (a), medium (c) or low (e) receptor expression levels) or the β_{3b}-AR (at high (b), medium (d) or low (f) receptor expression levels). Values represent means ± s.e.mean from 4–6 experiments. Data is presented as a percentage of the maximal responses to the highest concentration of CL316243 in each experiment in control treated cells. Note that PTX treatment increased responses only in cells expressing β_{3b}-AR.

Table 3 Effect of PTX (100 ng ml^{-1} 16 h) treatment on CL316243 mediated increases in extracellular acidification responses as assessed by cytosensor microphysiometer and cyclic AMP accumulation levels in cells expressing either the β_{3a}- or β_{3b}-AR at high, medium and low expression levels

	Extracellular acidification data					*Cyclic AMP accumulation data*				
	Control		*PTX treated*			*Control*		*PTX treated*		
	pEC_{50}	*Maximum (%)*	*pEC_{50}*	*Maximum (%)*	n	*pEC_{50}*	*Maximum (pmol/well)*	*pEC_{50}*	*Maximum (pmol/well)*	n
β_{3a}-AR										
High	10.79 ± 0.07	100	10.57 ± 0.10	108 ± 4	6	9.74 ± 0.15	347 ± 27	9.85 ± 0.15	298 ± 22	5
Medium	9.79 ± 0.05	100	9.69 ± 0.11	111 ± 5	6	9.16 ± 0.27	248 ± 15	9.17 ± 0.17	249 ± 11	4
Low	9.09 ± 0.09	100	9.25 ± 0.20	97 ± 8	4	7.57 ± 0.24	45 ± 3	7.68 ± 0.47	48 ± 6	4
β_{3b}-AR										
High	10.57 ± 0.05	100	10.11 ± 0.09	134 ± 5***	6	9.87 ± 0.10	247 ± 12	9.46 ± 0.25	496 ± 72***	5
Medium	9.88 ± 0.11	100	9.43 ± 0.13	139 ± 8***	5	8.97 ± 0.39	244 ± 27	8.76 ± 0.18	316 ± 19***	4
Low	9.12 ± 0.13	100	8.98 ± 0.17	153 ± 11***	5	7.76 ± 0.33	43 ± 4	7.61 ± 0.33	61 ± 6*	4

Agonist potency values are mean pEC_{50} values ± s.e.mean for *n* experiments. Maximal responses are expressed as either a percentage of response produced in control treated cells defined as 100% (cytosensor microphysiometer studies) or in cyclic AMP produced (pmol per well) for cyclic AMP accumulation studies. ***Indicates $P<0.001$, *indicates $P<0.05$ determined by two-way ANOVA of concentration-response curves produced in β_{3b}-AR cells treated or untreated with PTX.

β_{3b}-AR maximal response 75.16±4.95%; two-way ANOVA ***$P<0.0001$). PTX treatment (100 ng ml^{-1} 16 h) increased responses in cells expressing the β_{3b}-AR (two-way ANOVA ***$P<0.001$ high and medium expressing clones, *$P<0.05$ low expressing clones) while not affecting CL316243 responses in cells expressing the β_{3a}-AR (two-way ANOVA n.s.) at all levels of receptor expression examined (Figure 3; Table 3). CL316243 (10 μM) did not increase cyclic AMP levels in untransfected CHO-K1 cells (Figure 4).

Signalling pathways contributing to Erk1/2 phosphorylation

All experiments examining CL316243 mediated increases in Erk1/2 phosphorylation levels were conducted in high expressing cells. CL316243 caused phosphorylation of Erk1/2 in a concentration-dependent manner (Figure 5) with pEC_{50} values of 7.28±0.26 and 7.36±0.24 in cells expressing the β_{3a}- or β_{3b}-AR respectively ($n=3$; two-way ANOVA n.s.), while having no effect in untransfected CHO-K1 cells (Figure 5). PTX (100 ng ml^{-1} 16 h) treatment had no significant effect on CL316243 (10 μM) mediated increases in Erk1/2 phosphorylation in cells expressing either the β_{3a}- or β_{3b}-AR (Student's t-test n.s.). In cells expressing either receptor, the increase in Erk1/2 phosphorylation by CL316243 was not affected by treatment with H89 (PKA inhibitor, 10 μM, 1 h (Student's t-test n.s.), no effect also at 50 μM (data not shown)), but was inhibited by LY294002 (PI3Kγ inhibitor, 10 μM, 1 h; Student's t-test *$P<0.05$), PP2 (c-Src inhibitor, 10 μM, 1 h; Student's t-test *$P<0.05$), genistein (tyrosine

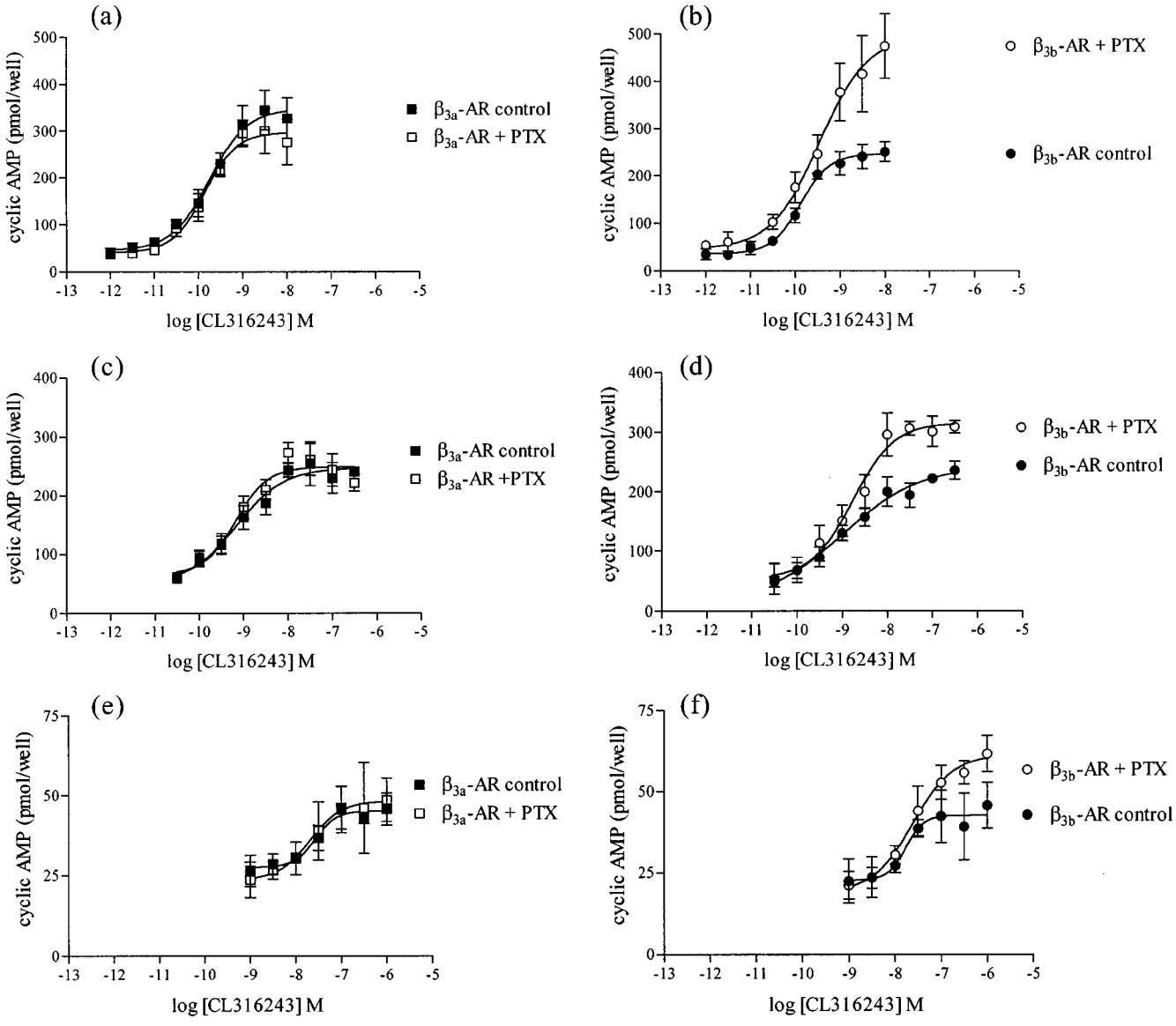

Figure 3 Effect of PTX (100 ng ml^{-1} 16 h) treatment on CL316243 mediated increases in cyclic AMP accumulation levels in cells expressing either the β_{3a}-AR (at high (a), medium (c) or low (e) levels of expression) or the β_{3b}-AR (at high (b), medium (d) or low (f) levels of expression). Values represent means±s.e.mean obtained from 4–5 experiments performed in duplicate. Data is presented as the amount of cyclic AMP accumulated (pmol per well) following CL316243 treatment. Note that PTX treatment increased maximal cyclic AMP responses only in cells expressing β_{3b}-AR. Note the difference in y-axis scale.

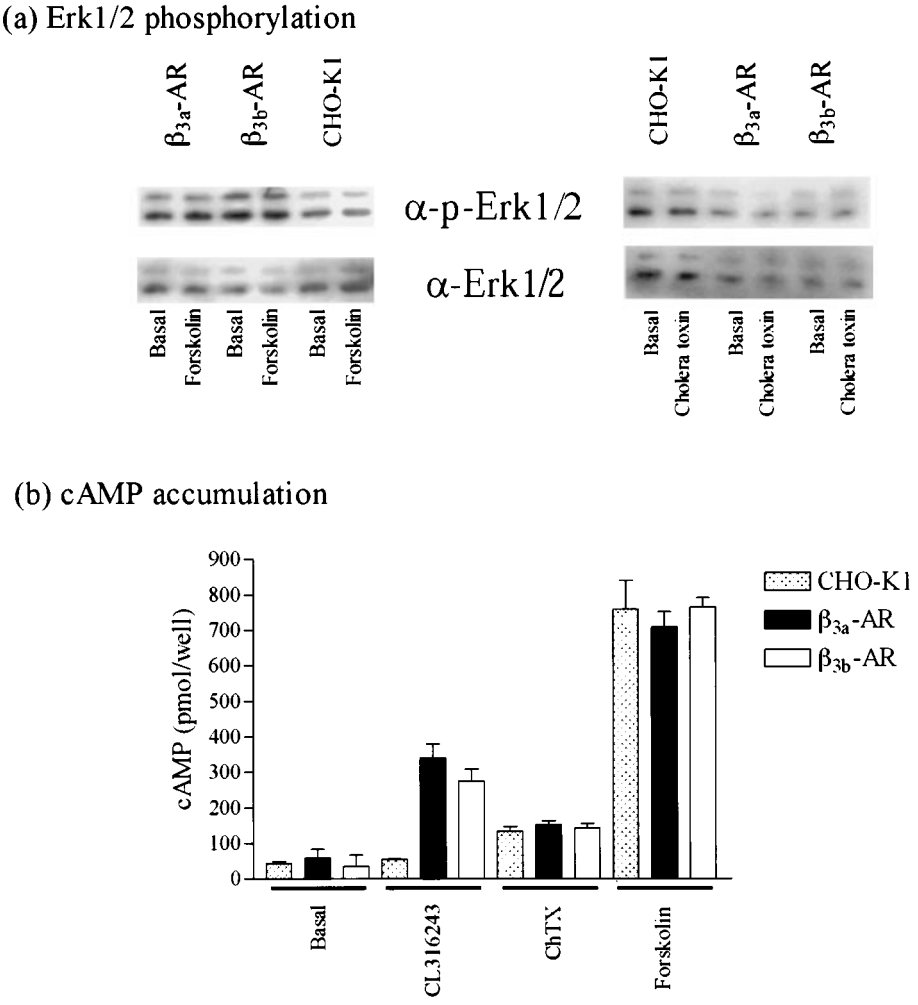

Figure 4 Effect of cholera toxin and forskolin treatment on (a) Erk1/2 phosphorylation and (b) cyclic AMP accumulation in transfected and untransfected CHO-K1 cells. Values represent means±s.e.mean obtained from three experiments performed in duplicate. Immunoblots are representative of three experiments performed in duplicate.

kinase inhibitor, 50 μM, 1 h; Student's *t*-test $^*P<0.05$) (Figure 6) and PD98059 (MEK inhibitor, 50 μM, 1 h, data not shown; Student's *t*-test $^*P<0.05$). Treatment of untransfected or β_{3a}- or β_{3b}-AR transfected CHO-K1 cells with forskolin (10 μM, 5 min) or cholera toxin (2 μg ml^{-1}, 90 min) produced no increase in Erk1/2 levels, although forskolin (10 mM, 30 min) and cholera toxin (2 μg ml^{-1}, 90 min) markedly increased cyclic AMP accumulation in these cells (Figure 4). Cyclic AMP accumulation experiments were performed in parallel with Erk1/2 phosphorylation studies to assess the effects of these treatments on cyclic AMP levels. LY294002, H89, PP2, PD98059 and genistein treatment had no significant effect on the ability of CL316243 to increase cyclic AMP levels in cells expressing either receptor (data not shown), while PTX treatment increased cyclic AMP levels in cells expressing the β_{3b}-AR but not the β_{3a}-AR.

Discussion

Alternate splicing of genes encoding GPCRs results in isoforms differing at their C-terminus that share similar pharmacological properties, but may show differences in distribution profiles and signalling properties. Here, we compare the pharmacology and signalling properties of mouse β_3-AR splice variants (Evans *et al*., 1999) expressed in CHO-K1 cells. There was no difference in the affinity of β-AR ligands between the β_{3a}- and β_{3b}-AR, consistent with the isoforms differing at the C-terminus and not in the transmembrane regions that form the β_3-AR ligand-binding pocket (Guan *et al*., 1995; Gros *et al*., 1998; Granneman *et al*., 1998). The rank order of potency of β-AR agonists assessed using the cytosensor microphysiometer was identical in cells expressing either receptor at high levels. The potency and efficacy of the β_3-AR selective agonist CL316243 was reduced with levels of receptor expression for both the β_{3a}- and β_{3b}-AR, as assessed by cyclic AMP accumulation and cytosensor microphysiometer studies. This observation is consistent with standard drug-receptor interaction models, both in theoretical (Kenakin, 1995a) and practical applications (Wilson *et al*., 1996; McDonnell *et al*., 1998; Cordeaux *et al*., 2000). In the cytosensor microphysiometer, all β-AR agonists caused a larger maximal response with β_{3a}-AR compared to β_{3b}-AR, but the pEC_{50} values were similar at each receptor. This indicated that differential coupling of each receptor to intracellular signalling pathways and not

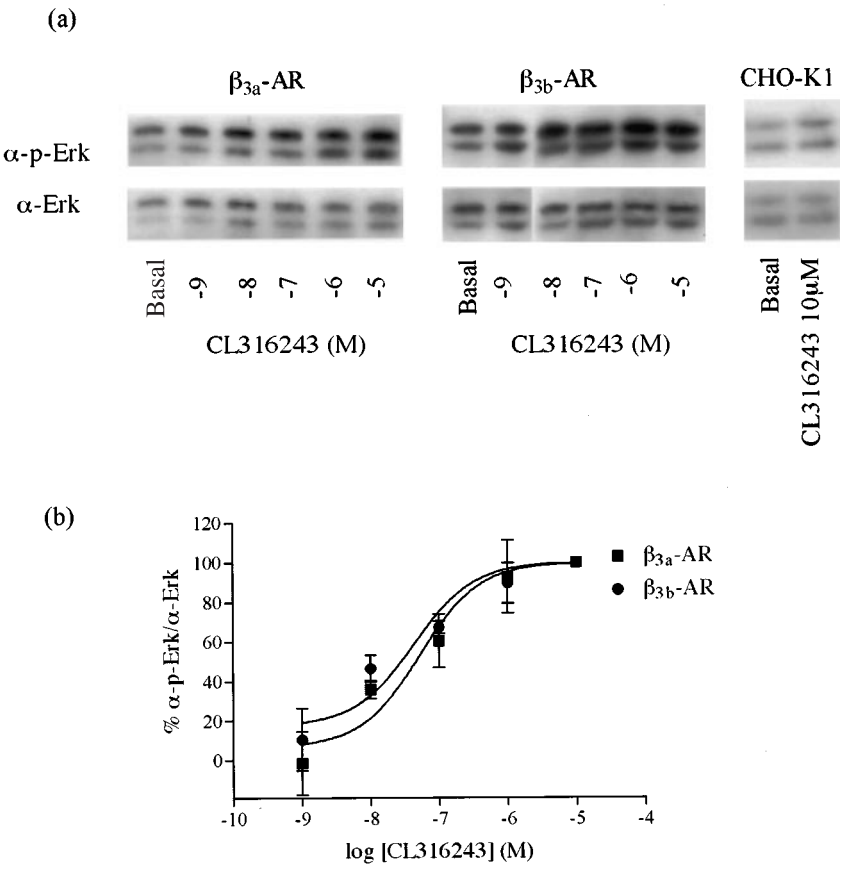

Figure 5 Concentration–response curve for Erk1/2 activation by CL316243 in high expression level cells expressing either the β_{3a}-AR or β_{3b}-AR. Values represent means ± s.e.mean obtained from three experiments performed in duplicate. Data is presented as percentage of the maximal responses to 10μM CL316243 at each receptor.

differences in expression levels is responsible for the observed difference in maximal response.

β_3-ARs are known to couple to G_s and activate adenylate cyclase, increasing intracellular cyclic AMP levels. The β_3-AR agonist CL316243 increased intracellular cyclic AMP in a concentration-dependent manner in β_{3a}- or β_{3b}-AR cells, suggesting coupling of both receptors to G_s. However, as with the cytosensor microphysiometer, maximal cyclic AMP responses in β_{3b}-AR cells were lower (by 25–30%) than in β_{3a}-AR cells. It is known that the β_3-AR can couple to G_i (Chaudhry *et al*., 1994; Soeder *et al*., 1999) and in 3T3-F442A adipocytes endogenously expressing β_3-ARs, PTX treatment enhances cyclic AMP accumulation in response to CL316243 (Soeder *et al*., 1999). Brown adipocytes in primary culture also show a 50% increase in BRL37344-stimulated cyclic AMP production in the presence of PTX (Lindquist *et al*., 2000). We find that CL316243-mediated cyclic AMP or cytosensor responses are increased following PTX treatment in β_{3b}-AR but not β_{3a}-AR cells. The protocol used for PTX treatment in the present studies has been used extensively in previous studies with β_3-AR (Cao *et al*., 2000; Gerhardt *et al*., 1999; Soeder *et al*., 1999) and was clearly effective in demonstrating the difference between β_{3a}- and β_{3b}-AR. Facilitation of cyclic AMP responses in 3T3-F442A cells or brown adipocytes by PTX treatment may therefore indicate that a fraction of the response to agonist treatment is mediated by endogenous β_{3b}-ARs. Although unphysiological promiscuous receptor-second messenger coupling can occur at high receptor expression levels (Eason *et al*., 1992; Kenakin, 1995a, b; Cordeaux *et al*., 2000), it is unlikely to be a factor in the medium (~500 fmol mg protein^{-1}) and low (~100 fmol mg protein^{-1}) expressing cells used here since these receptor levels are similar to physiological levels reported in rat white adipocytes (~400–600 fmol mg protein^{-1}; Germack *et al*., 1996), rat brown adipocytes (~430 fmol mg protein^{-1}; Sillence *et al*., 1993), and mouse ileum (~60 and ~150 fmol mg protein^{-1}; Hutchinson *et al*., 2000 and unpublished data respectively).

We find that treatment of CHO-K1 cells expressing either β_{3a}- or β_{3b}-ARs with CL316243 increases Erk1/2 phosphorylation by 2 fold in a concentration-dependent manner, similar to other studies using CL316243 (Gerhardt *et al*., 1999; Soeder *et al*., 1999; Cao *et al*., 2000). The concentration-response curve for Erk1/2 activation by CL316243 in CHO-K1 cells expressing either receptor is shifted significantly to the right compared to cyclic AMP accumulation responses (over 100 fold shift), compared to 30 fold shift reported by Soeder *et al*. (1999). This suggests that activation of G_s/adenylate cyclase/cyclic AMP occurs at low concentrations of agonist, but that Erk1/2 phosphorylation becomes significant at high levels of receptor occupancy. The present experiments showed that there was no difference of Erk1/2

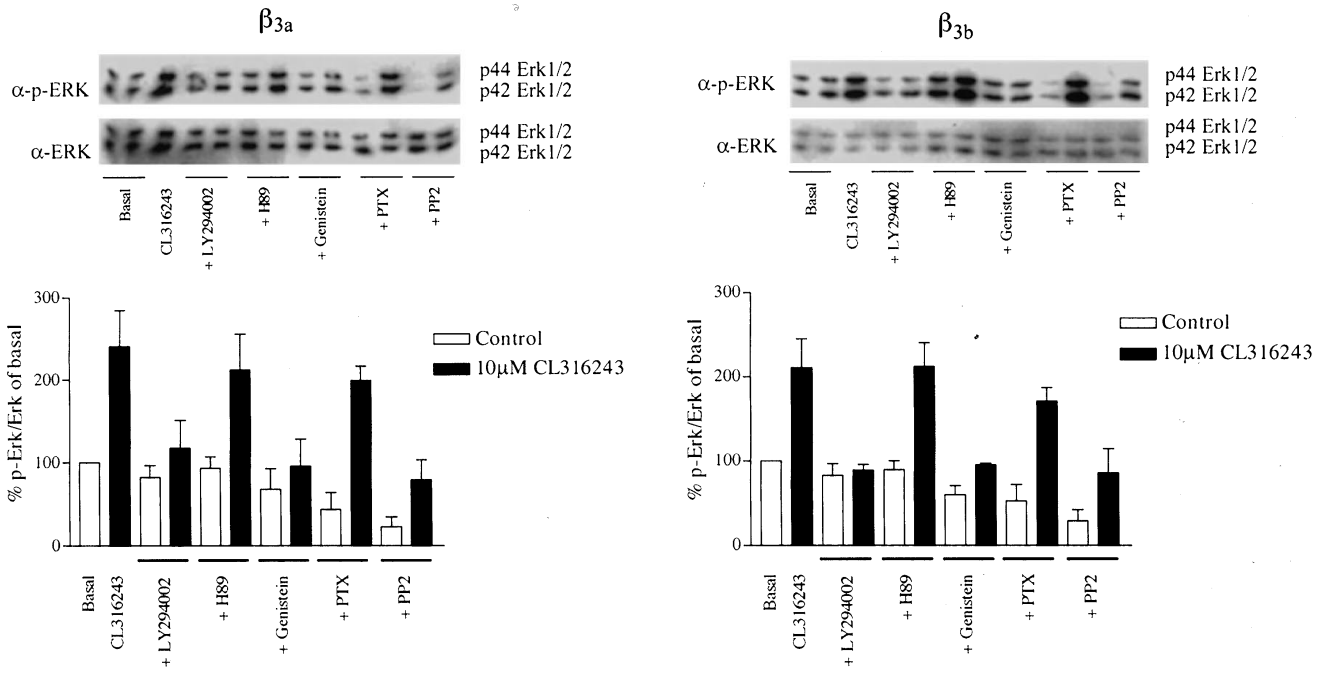

Figure 6 Effect of signal transduction inhibitors on Erk1/2 phosphorylation levels following CL316243 (10 μM, 5 min) stimulation in cells expressing either the β_{3a}-AR (left panels) or the β_{3a}-AR (right panels). Cells were treated either with PTX (100 ng ml^{-1} 16 h), or genistein (50 μM), or H89 (10 μM), or PP2 (10 μM), or LY294002 (10 μM) for 1 h. Values represent means$\pm$s.e.mean obtained from four experiments performed in duplicate. Data is presented is a percentage of the response in control cells. ADP ribosylation of G_i by PTX or PKA inhibition by H89 failed to inhibit Erk1/2 phosphorylation following agonist stimulation of cells expressing either β_{3a}- or β_{3b}-AR.

activation between the β_{3a}- or β_{3b}-AR. This would rule out an involvement of G_i in Erk1/2 activation, and in addition this was further supported by the observation that Erk1/2 activation was insensitive to treatment with PTX. Both forskolin and cholera toxin did not activate Erk1/2 in both untransfected and transfected CHO-K1 cells suggesting that Erk1/2 activation is not dependent upon cyclic AMP or G_s. The PKA inhibitor H89 was also ineffective in preventing the activation of Erk1/2 by the β_3-AR agonist CL316243 in cells transfected with either β_{3a}- or β_{3b}-AR. On the other hand activation of Erk1/2 was sensitive to the inhibitors of Src (PP2), PI3K (LY294002) and the general tyrosine kinase inhibitor genistein. The potential involvement of $G\beta\gamma$ subunits cannot be ruled out and the use of the C-terminal fragment of β-ARK may be helpful for future studies investigating the role of $G\beta\gamma$ subunits on Erk1/2 activation in this system. Hence the differences in the concentration-response curves observed for cyclic AMP accumulation and Erk1/2 activation may reflect a lack of involvement of $G\alpha$-proteins in the stimulation of Erk1/2 phosphorylation in this system.

Phosphorylation of Erk1/2 occurs in cell systems expressing endogenous or transfected β_2- or β_3-ARs. The mechanism by which this occurs, however, differs according to the receptor and the cell type. β_2-AR mediated activation of Erk1/2 is linked to receptor phosphorylation, binding of β-arrestin, and subsequently binding of c-Src to the β-arrestin (Luttrell *et al*., 1999; Maudsley *et al*., 2000). There is no phosphorylation of the β_3-AR with agonist stimulation (Liggett *et al*., 1993) and this receptor is unable to bind β-arrestin (Cao *et al*., 2000). However, activated β_3-ARs bind c-Src directly *via* four motifs (PXXP) in the third intracellular loop and the C-terminus (Cao *et al*., 2000). Mutation of proline residues in these motifs prevents both c-Src binding and Erk1/2 phosphorylation. Activation of the Erk1/2 pathway *via* β_3-ARs in mouse adipocytes is inhibited by the selective c-Src inhibitor, PP2 (Lindquist *et al*., 2000). We also found that Erk1/2 phosphorylation was inhibited by PP2 in β_{3a}- or β_{3b}-AR CHO-K1 cells.

In most cell systems, Erk1/2 phosphorylation is inhibited by PTX or the C-terminus of β-ARK, which sequesters $G\beta\gamma$ subunits. The involvement of G_i or $G\beta\gamma$ subunits derived from G_i in Erk1/2 phosphorylation differs between brown and white adipocytes. In brown adipocytes a G_s/PKA-dependent rap1/B-raf pathway mediates β_3-AR stimulation of Erk1/2 activation (Lindquist *et al*., 2000), while in white adipocytes H89 has no effect and there is clear inhibition by PTX, indicating a requirement for G_i (Soeder *et al*., 1999). Activation of Erk1/2 by human β_3-ARs expressed in HEK293 (Soeder *et al*., 1999) or CHO-K1 (Gerhardt *et al*., 1999) cells is sensitive to PTX but not H89, indicating a role for G_i but not G_s. We found that H89 had no effect on Erk1/2 phosphorylation in β_{3a}- or β_{3b}-AR CHO-K1 cells. Also, treatment of untransfected or transfected CHO-K1 cells with forskolin or cholera toxin failed to increase Erk1/2 levels. This suggests that activation of Erk1/2 in these cells is not dependent upon increases in cyclic AMP, consistent with some studies (Gerhardt *et al*., 1999; Ai *et al*., 1999) but not others (Verheijen & Defize, 1997). We have demonstrated coupling of the β_{3b}-AR to G_i, but unlike CHO-K1 cells expressing the human β_3-AR, PTX had no effect on Erk1/2 phosphorylation in response to CL316243 in β_{3b}-AR CHO-

K1 cells. These differences in the PTX sensitivity of Erk1/2 phosphorylation may reflect sequence differences between the mouse and human β_3-ARs. Whereas the mouse β_3-AR has four PXXP motifs for the binding of c-Src, the human receptor has only two. Weaker association of c-Src with the human β_3-AR may increase the dependence of the MAPK signalling pathway on $G_i/G\beta\gamma$. Finally, in CHO-K1 cells expressing human (Gerhardt *et al*., 1999) or mouse β_{3a}- or β_{3b}-ARs, Erk1/2 phosphorylation is blocked by LY294002 (PI3K inhibitor). PI3Ks are stimulated by $G\beta\gamma$ subunits (Gerhardt *et al*., 1999), although this is inconsistent with our observation that Erk1/2 phosphorylation was unaffected by PTX. So while a PI3K pathway is present, the mechanism of activation remains to be elucidated. Recent reports show that $G\beta\gamma$ subunits from G_s can couple to other intracellular signalling events such as isoprenaline-mediated β_2-AR inhibition of NADPH-dependent H_2O_2 generation in human adipocytes (Krieger-Brauer *et al*., 2000). Thus mechanisms involving G_s may not universally require elevation of cyclic AMP and activation of PKA.

The β_{3a}- and β_{3b}-AR isoforms differ at the C-terminus. Motifs present in the C-termini of GPCRs, including PDZ-binding and PSD-95-binding domains in the β_2- and β_1-AR C-terminus respectively (Cao *et al*., 1999; Hu *et al*., 2000) are important for interactions with a range of targeting and recycling proteins. The C-terminal motifs in both these receptors show complete conservation amongst nine mammalian species, indicative of their critical importance. In contrast, the final amino acid residues following the putative palmitoylation site (Cys361) in the C-terminus of β_3-ARs are much less conserved amongst the nine mammalian species examined. There is only one conserved PXXP motif that is consistently present in the β_3-AR C-terminus. At the moment it is not possible to tell whether the secondary structure of the β_3-AR C-terminus facilitates protein–protein interactions. Our hypothesis is that the altered amino acid sequence of the β_{3b}-AR affects the conformation of the proximal region of the C-terminus and the third intracellular loop due to hydrogen bonding or other interactions involving amino acids uniquely present in the β_{3b}-AR, or because of an additional cysteine in the β_{3b}-AR that may affect palmitoylation of Cys361 and hence formation of the 'fourth intracellular loop'.

The present study demonstrates that the β_{3a}- and β_{3b}-AR share similar affinity and potency for all β-AR ligands examined. However, in cyclic AMP and cytosensor microphysiometer studies, the β_{3b}-AR coupled to both G_s and G_i, while the β_{3a}-AR coupled solely to G_s. Increases in Erk1/2 phosphorylation following β_{3a}- or β_{3b}-AR activation were independent of receptor coupling to G_i, and involved a mechanism that includes activation of PI3Kγ and c-Src. Activation of Erk1/2 signalling by β-ARs and other GPCRs is clearly a complex process modulated by multiple signalling proteins that differ between cell types.

This work was supported by the National Health and Medical Research Council of Australia, the Tore Nilsons Stiftelse for Medicinsk Forskening (Sweden) and the Jeanssonska funds (Sweden). D.S. Hutchinson is a Monash University Postgraduate Scholar and is supported by the Monash University Postgraduate Publications Award. The authors wish to record their appreciation for technical assistance provided by Maria Papaioannou and Johanna Lindquist for parts of this study.

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(Received October 16, 2001
Revised January 23, 2002
Accepted February 5, 2002)