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Influence of receptor number on the stimulation by salmeterol of gene transcription in CHO-K1 cells transfected with the human β_2-adrenoceptor

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1 The β_2-agonist salmeterol is a potent relaxant of airway smooth muscle with a long duration of action. Previous studies of cyclic AMP accumulation, however, have indicated that salmeterol is a low efficacy β_2-agonist when compared to isoprenaline. Here we have compared the properties of salmeterol and isoprenaline as stimulants of gene transcription in CHO-K1 cells transfected with the human β_2-adrenoceptor to different levels (50 and 310 fmol mg protein^{-1}).

2 Gene transcription was monitored using a secreted placental alkaline phosphate (SPAP) reporter gene under the transcriptional control of six cyclic AMP response element (CRE) sequences.

3 In the lower expressing cells (CHO-β_2/6), salmeterol produced a maximal cyclic AMP response that was only 22% that of that obtained with isoprenaline. In contrast in the higher expressing cells (CHO-β_2/4), the two maxima were of similar magnitude.

4 Salmeterol was a more potent stimulant of gene transcription, producing the same maximal response as isoprenaline in both cell lines. Furthermore, in the CHO-β_2/4 cells, Salmeterol was 50 fold more potent as a stimulant of SPAP secretion than of cyclic AMP accumulation. In contrast, isoprenaline was 24 fold less sensitive as a stimulant of SPAP secretion than of cyclic AMP accumulation. In the presence of serum (10%), the effects of both salmeterol and isoprenaline on gene transcription were augmented.

5 These data suggest that the low efficacy and/or long duration of action of salmeterol, favours a potent stimulation of gene transcription when compared to more efficacious but shorter-lived agonists such as isoprenaline.

Keywords: Gene transcription; β_2-adrenoceptor; cyclic AMP response element; CHO-K1 cells; salmeterol; cyclic AMP; receptor number; reporter gene; secreted alkaline phosphatase

Introduction

The β_2-selective agonist salmeterol is a potent relaxant of airway smooth muscle (Nials *et al*., 1993; Coleman *et al*., 1996). However, unlike many other β_2-agonists, the bronchodilation produced by salmeterol is very prolonged (>12 h) and resistant to repetitive washout (Nials *et al*., 1993; Coleman *et al*., 1996). A striking feature of salmeterol's effect on smooth muscle is that it reappears following β_2-receptor antagonism (by a hydrophillic β_2-antagonist such as sotolol) and subsequent washout of the antagonist (Nials *et al*., 1993; Coleman *et al*., 1996; Clark *et al*., 1996). These observations led to the hypothesis that the saligenin moiety of the salmeterol molecule binds to the active site of the β_2-adrenoceptor while the hydrophobic tail of the molecule binds to a secondary exosite (Coleman *et al*., 1996; Jack, 1991).

Studies in which the duration of action of salmeterol was compared on β_2- and β_1-adrenoceptors in C6 glioma cells (which possess both receptors) suggested that the exosite was part of the β_2-adrenoceptor, rather than a consequence of any partitioning of this highly lipophillic molecule within the bulk lipid of the plasma membrane (McCrea & Hill, 1996). Site-directed mutagenesis studies have confirmed that the exosite binding domain is located within a limited 10 amino acid region of transmembrane spanning domain 4 (TM4) of the human β_2-adrenoceptor (residues 149–158) (Green *et al*., 1996). Furthermore, substitution of residues 152–156 (Val-lle-lle-Leu-Met) of the β_2-adrenoceptor into the corresponding positions of the β_1-adrenoceptor, confers sustained salmeterol-mediated β_1-agonist activity (Green *et al*., 1996).

We have previously shown that salmeterol can produce sustained increases in cyclic AMP accumulation in cultured B50 neuronal cells and C6 glioma cells, whereas agonists such as isoprenaline and formoterol are much shorter lived (McCrea & Hill, 1993, 1996). It is notable, however, that salmeterol behaves as a low efficacy agonist on cyclic AMP accumulation in these cells (McCrea & Hill, 1993, 1996; Green *et al*., 1996). A similar observation has been made in L cells where the long duration of activation of adenylyl cyclase activity by salmeterol is also associated with a relatively low efficiency of coupling to the effector enzyme (Clark *et al*., 1996). This sustained effect on cyclic AMP accumulation with salmeterol is surprising since prolonged agonist activation of β_2-adrenoceptors by isoprenaline can lead to rapid desensitization (Inglese *et al*., 1993; Lefkowitz, 1993; Yuan *et al*., 1994; Freedman & Lefkowitz, 1996), although it is possible that the lower efficacy of salmeterol reduces the susceptibility to desensitization (Clark *et al*., 1996; Inglese *et al*., 1993; Yuan *et al*., 1994; Benovic *et al*., 1988).

In addition to mediating responses such as smooth muscle relaxation, increases in the intracellular levels of cyclic AMP also induce transcription of a variety of genes through activation of cyclic AMP response elements (CREs) *via* the action of a CRE-binding protein (CREB) (Montminy *et al*., 1990; Lalli & Sassone-Corsi, 1994; Swope *et al*., 1996). The

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CRE in many genes, notably that for somatostatin, α-chorionic gonadotrophin and corticotrophin releasing hormone (Montminy *et al*., 1990; Guardiola-Diaz *et al*., 1994) is made up of the palindromic sequence TGACGTCA, although variations in this are common (Montminy *et al*., 1990). This sequence when positioned upstream of a reporter gene, such as luciferase, can be used to monitor the effects of cyclic AMP elevating agents on gene transcription (Guardiola-Diaz *et al*., 1994; Migeon *et al*., 1995; Pepperl & Regan, 1993). In the present study we have used a reporter construct containing the cDNA for human placental secreted alkaline phosphatase (SPAP; Berger *et al*., 1988), under the transcriptional control of six CRE sequences, to compare the effects of isoprenaline and salmeterol on gene expression in two cell lines expressing the human β_2-adrenoceptor at different levels.

Methods

Construction of pCIN/β_2

All DNA manipulations were performed using standard methods unless otherwise described (Sambrook *et al*., 1989). The cDNA for the human β_2-adrenoceptor (Genbank accession no. M25269) was subcloned into the *Eco*RI site of the bicistronic mammalian expression vector pCIN1 (Rees *et al*., 1996). The vector was linearized by digestion with SspI prior to transfection.

Construction of p6CRE-sPAP-Hyg

The synthetic oligonucleotide 5′-CCAGAAGCCTACGTAGGCGTCGACCTCCTTGGC**TGACGTCA**GTAGAGAGATCCCAT**TGACGTCA** TACTGAGA CGTAGATCTC CAT**TGACGTCA**AGGAGACTCGAGGCTCCATCGCAGTGATCG-3′ containing three copies of the consensus cyclic AMP response element (CRE; bold) (Guardiola-Diaz *et al*., 1994) and *Sal*I and *Xho*I restriction sites (underlined), was used as the template in a PCR reaction using flanking primers 5′-CCAGAAGCCTACGTAGGCGTC-3′ and 5′-CGATCACTGCGATGGAGCCTC-3′ to amplify a 123 bp fragment containing three copies of the consensus CRE. The PCR product was digested with *Sal*I and *Xho*I and inserted between the *Sal*I and *Xho*I sites of pBluescript-KS (Stratagene) to generate pBS/3CRE. Following sequencing the 3CRE element was excised by digestion with *Sal*I and *Xho*I and cloned into the unique *Sal*I site of the vector pTK-sPAP, a gift from Dr D.M. Wallace, Glaxo Wellcome Research and Development, containing the secreted placental alkaline phosphatase (sPAP) gene downstream of the minimal Herpes Simplex Virus Thymidine Kinase (HSV-TK) promoter (−110 to +101), to generate p3CRE/sPAP. This vector was subsequently digested with *Sal*I and a second copy of the 3CRE element was inserted to generate p6CRE-sPAP.

The construction of the expression vector pcDNA4, a derivative of pcDNA3 (Invitrogen) has been described (Rees *et al*., 1996). The CMV promoter was removed from pcDNA4 by digestion of this plasmid with *Nru*I and *Eco*RI. This was followed by insertion of the synthetic linker created following annealing of the oligonucleotides 5′-CCGTCGACAGTCAGCTAGCG-3′ and 5′-AATTCGCTAGCTGACTGTCGACGG-3′ which encode *Sal*I and *Nhe*I restriction sites (underlined) to generate pMCS-neo. To replace the neomycin phosphotransferase gene with that for hygromycin aminotransferase the vector pORFEX10-Hm24 (also a gift from Dr D. Wallace, Genomics Unit, Glaxo Wellcome Research and Development) was digested with *Bgl*II and *Xba*I to release a fragment containing the CMV promoter and the hygromycin selection marker and this was inserted between the *Bst*BI and *Avr*II sites of pMCSneo, deleting the SV40 origin and early promoter and the neomycin selection marker, to generate pMCShyg.

To generate an all-in-one vector suitable for the derivation of stable cell lines, p6CRE-sPAP was digested with *Spe*I and *Xba*I to release a fragment containing the 6CRE-sPAP sequences and this was inserted into the *Nhe*I site of pMCShyg to generate p6CRE-sPAP-Hyg: The structure of the synthetic 6CRE promoter is shown in Figure 1.

Cell culture

Chinese Hamster Ovary (CHO) cells were grown in Dulbecco's modified Eagles medium/Nutrient mix F12 (1 : 1) supplemented with 2 mM L-glutamine and 10% foetal calf serum at 37°C in an atmosphere of 5% CO_2:95% air.

Generation of cell lines

For transfection with the human β_2-adrenoceptor, cells were plated at 1% confluence in a 9 cm diameter tissue culture dish and transfected with 10 μg linearized pCIN/β_2 using the calcium phosphate transfection kit (Invitrogen, NV Leek, The Netherlands). Following clonal selection in the presence of 1 mg ml^{-1} G418 (Life Technologies) cells were screened for receptor expression by ligand binding and measurement of cyclic AMP. Two clonal lines (CHO.β_2/4 and CHO.β_2/6) were selected for further study. These two cell lines were then stably transfected with a vector (p6CRE-sPAP-Hyg) encoding for the reporter gene, secreted placental alkaline phosphatase (SPAP). Cells were grown to 60% confluence in 6-well cluster dishes and incubated in serum free medium with 10 μg DNA/well in the presence of 10 μl Transfectam for 4 h at 37°C. Transfected cells were selected in the presence of 200 μg ml^{-1} hygromycin.

[^{125}I]-iodocyanopindolol (lCYP) binding

Radioligand binding studies were based on a method by Neil *et al*. (1997) and were performed on membranes prepared from CHO.β_2/4 and CHO.β_2/6 cells. Confluent cultures were scraped into phosphate buffered saline (PBS), centrifuged at 1000 r.p.m. for 10 min and resuspended in 50 mM Tris (pH 7.4) with 1 mM EDTA. This suspension was homogenized in a hand held homogenizer and centrifuged at 18,000 × *g* for 10 min. Pellets were resuspended in Tris EDTA buffer and recentrifuged. The resulting pellet was resuspended in Tris EDTA to a protein concentration of 1 mg ml^{-1}. Membranes (10–20 μg protein)

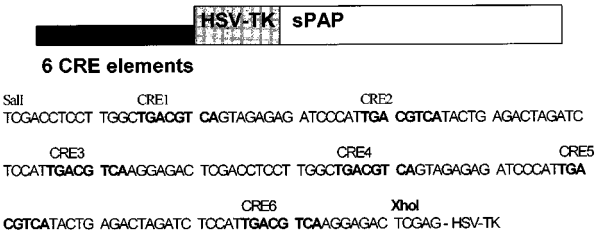

Figure 1 Structure of the 6CRE-sPAP reporter gene. Six copies of the consensus cyclic AMP response element were subcloned upstream of the minimal HSV-TK promoter (−110 to +101) and used to promote expression of the sPAP reporter gene (see Methods for details).

were incubated with 25–200 pM [^{125}I]-lCYP) in a final volume of 250 μl for 1 h at 37°C. Reactions were stopped by dilution with Tris ascorbate buffer (50 mM: 2 mM pH 8) and rapid filtration over Whatman GF/B filters. Bound [^{125}I]-lCYP was measured by gamma counting. Non-specific binding was determined in the presence of 1 μM propranolol. Protein content was determined by the method of Lowry *et al*. (1951).

Cyclic AMP accumulation

Cyclic AMP assays were performed as described previously (McCrea & Hill, 1996). Briefly, confluent cells grown in 24 well cluster dishes were prelabelled with [^{3}H]-adenine for 2 h at 37°C in 1 ml well^{-1} Hanks balanced salt solution (HBH; containing 20 mM HEPES, pH 7.4). Cells were washed and then incubated for 30 min in 1 ml well^{-1} HBH containing the phospodiesterase type IV inhibitor rolipram (100 μM; Donaldson *et al*., 1988). Unless otherwise stated, rolipram (100 μM) was included in all subsequent incubations. Where used, antagonists were added to the cell monolayer during this 30 min preincubation period. Agonists (in 10 μl HBH) were then added to the assay system, and the incubation was continued for 10 min. Reactions were stopped by the addition of 50 μl concentrated HCl. [^{3}H]-cyclic AMP was separated from other [^{3}H]-nucleotides by sequential dowex and alumina chromatography as previously described (Donaldson *et al*., 1988). Samples were measured by liquid scintillation counting. [^{3}H]-cyclic AMP production was expressed as a percentage conversion from total [^{3}H]-adenine.

Measurement of SPAP

Assays were performed on confluent cells grown in 24 well cluster dishes. The medium was aspirated and replaced with fresh complete (with 10% FCS) DMEM/F12 medium (1 ml well^{-1}) containing the appropriate concentration of agonist or, where used, antagonist. Antagonists were preincubated for 30 min prior to the addition of agonist. Reactions were allowed to proceed at 37°C for 5 or 24 h. Medium was removed from the 5 h incubation and replaced with 300 μl well^{-1} fresh medium. The reaction was allowed to proceed for a further 1 h when the supernatant medium was transferred to eppendorf tubes. For the 24 h assay samples were collected to measure the generation of SPAP over the whole 24 h. In some experiments, cells were incubated in serum-free DMEM/F12 medium for 24 h prior to addition of agonists and/or serum (10%) for a further 5 h. Endogenous alkaline phosphatases were inactivated by heating the samples to 65°C for 30 min. SPAP was measured in 10 μl (24 h assay) or 20 μl (5–6 h assay) by hydrolysis of *p*-nitrophenol phosphate monitored at 405 nm after 1 h (24 h assay) or 2 h (5–6 h assay) incubation at 37°C (Cullen & Malim, 1992). Data were expressed either as percentage of the basal secretion or converted to SPAP concentration in mU ml^{-1} using the following equation (Cullen & Malim, 1992):

$$[\text{SPAP}]\ (\text{mU ml}^{-1}) = \frac{A}{tx18.5xV}$$

Where A is the measured optical density at 405 nm, t is the time with substrate (60 or 120 min) and V is the volume of sample (ml).

Data analysis

Cyclic AMP data were corrected for interwell variability in cell number and recovery of tracer [14C]-cyclic AMP, from Dowex alumina chromatography. Agonist and antagonist concentration-response curves were fitted to a four parameter logistic equation through computer-assisted curve fitting (Prism 2, GraphPad Software). The equation fitted was:

$$\text{Response} = E_{min} + \frac{(E_{max} - E_{min})}{(1 + 10^{(\log EC50 - X).n})}$$

where E_{MIN} is the basal response, E_{MAX} is the maximal stimulation, X is the agonist concentration and n is the Hill coefficient.

Data represent means ± s.e.mean of triplicate or quadruplicate determinations in varying numbers of experiments (actual number given in the text). Statistical analysis was performed with the use of Student's *t*-tests and two-way analysis of variance.

Materials

pBluescript-KS was obtained from Stratagene Ltd Cambridge; G418 from Gibco Life Technologies, Paisley, U.K.; Transfectam from Promega U.K. Ltd, Chilworth Research Centre, Southampton, Harts., U.K.; hygromycin from Boehringer Mannheim; [^{125}I] lodocyanopindolol, [^{3}H]-adenine and [^{14}C]-cyclic AMP from New England Nuclear Life Science Products, Hounslow, Essex, U.K. All other chemicals were obtained from either Sigma Chemicals, Dorset, U.K. or Fisher Scientific, Leics., U.K.

Results

Receptor expression and cyclic AMP accumulation

Following initial subcloning of the cells expressing the cDNA for the human β_2-adrenoceptor, two cell lines (CHO-β_2/4; CHO-β_2/6) were selected which had different levels of receptor expression (Table 1). These cell lines were used for measurement of both cyclic AMP accumulation and SPAP secretion. The higher expressing cell line CHO-β_2/4 (310 fmol.mg protein^{-1}) produced a larger maximal increase in [^{3}H]-cyclic AMP accumulation in response to isoprenaline than did the lower expressing CHO-β_2/6 cell line (50 fmol.mg protein^{-1}) (Figure 2a). It was notable, however, that the basal level of [^{3}H]-cyclic AMP obtained in CHO-β_2/4 cells was an order of magnitude higher than that in the lower expressing cell line (Figure 2a; Table 2). One explanation for this is that the higher expression of β_2-adrenoceptors in CHO-β_2/4 cells has induced a degree of constitutive receptor activity (Chidiac *et al*., 1994; Samama *et al*., 1994; Milano *et al*., 1994; MacEwan *et al*., 1995). In order to test this, the effect of ICI 118551, a β_2-adrenoceptor-selective antagonist with inverse

Table 1 Specific ^{125}I-Iodocyanopindolol binding to CHO-K1 cells transfected with the human β_2-adrenoceptor

	B_{max} (*fmol mg protein*$^{-1}$)	K_D (pM)	n
CHO.β_2/4 cells	310 ± 70	92 ± 17	7
CHO.β_2/6 cells	50 ± 1	64 ± 20	3

^{125}I-Iodocyanopindolol was incubated with membrane preparations (10–20 μg tube^{-1}) in the absence and presence of 1 μM propranolol to determine specific binding. Protein concentrations were determined by the method of Lowry using bovine serum albumin as a control. Values represent means ± s.e.mean. n = the number of separate experiments.

agonist properties (Samama *et al*., 1994; Milano *et al*., 1994), on basal and isoprenaline-stimulated cyclic AMP accumulation was evaluated (Figure 2b). These data clearly show that ICI 118551 can markedly reduce basal [^{3}H]-cyclic AMP accumulation (Figure 2b), consistent with the presence of agonist-independent β_2-adrenoceptor activity.

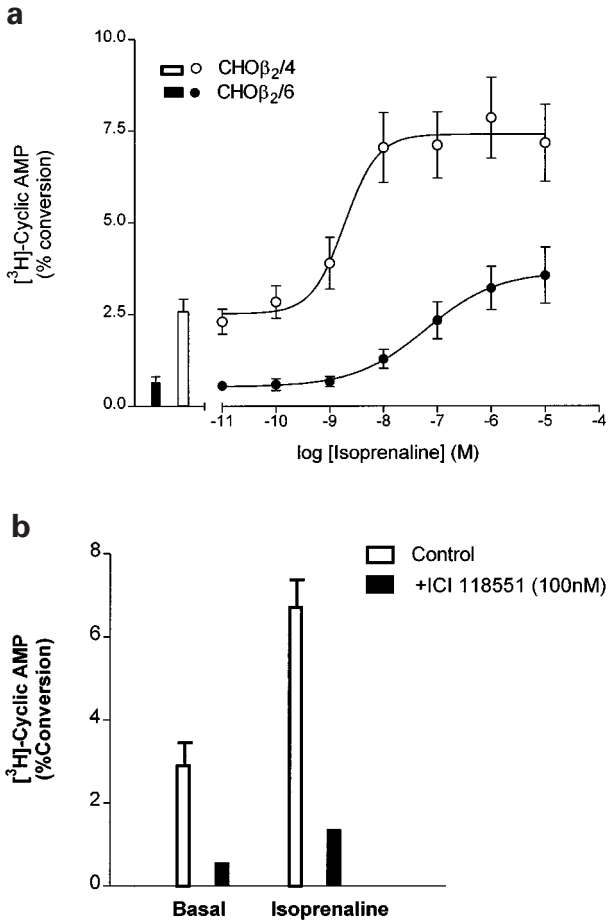

Figure 2 Isoprenaline-stimulated [^{3}H]-cyclic AMP accumulation in CHO-K1 cells expressing the human β_2-adrenoceptor. (a) cyclic AMP accumulation in cells expressing the human β_2-adrenoceptor at two different levels of expression. Values represent means$\pm$s.e.mean of triplicate determinations in three separate experiments. Bars show the basal responses in CHO-β_2/4 cells (open bar) and CHO-β_2/6 cells (filled bar). Data are expressed as a percentage of the total [^{3}H]-adenine in the cells. (b) The effect of the β_2-selective antagonist ICI 118551 on isoprenaline-stimulated and basal accumulation of [^{3}H]-cyclic AMP in CHO-β_2/4 cells. Values represent means$\pm$s.e.mean of triplicate determinations in a single experiment obtained under control conditions (open bars) and following 30 min pretreatment with ICI 118551 (100 nM; filled bars). Cells were stimulated with 100 nM isoprenaline for 20 min. Similar results were obtained in a repeat experiment.

A comparison of the agonist EC_{50} values for isoprenaline in the two cells indicate that, this β-agonist was *circa* 30 fold more potent in CHO-β_2/4 cells than it was in the CHO-β_2/6 cell line (Table 2; Figure 2a). The difference in EC_{50} values for salmeterol-induced cyclic AMP accumulation in the two cell lines was, however, much lower (ratio = 2.6 fold; Table 3). This is primarily because salmeterol acted as a partial agonist in CHO-β_2/6 cells (E_{MAX} = 22% of the response to 1 μM isoprenaline; Table 3; Figure 3) and the effect of the increased receptor expression was primarily to enable salmeterol to produce the same maximal response as the more efficacious agonist isoprenaline in CHO-β_2/4 cells (Figure 3; Table 3).

Isoprenaline-stimulated SPAP secretion

Concentration-response analysis of isoprenaline-stimulated SPAP secretion from both CHO-β_2/4 and CHO-β_2/6 cell lines (following 24 h agonist incubation) indicated that there was a similar difference in EC_{50} values, between the two cell lines, to that seen with cyclic AMP accumulation (25 fold; Figure 4a; Table 2), although there were differences in the absolute levels of SPAP accumulated in the extracellular medium (Table 2). Measurement of the SPAP secreted between 5–6 h, following isoprenaline administration, also showed that cells were much more sensitive to isoprenaline in the higher expressing CHO-β_2/4 cells (Figure 4b; Table 2).

The secretion of SPAP stimulated by isoprenaline was antagonized by the β_2-selective antagonist ICI 118551 (Figure 5a) and the non-selective β-antagonist propranolol (Figure 5b). It was notable that ICI 118551 reduced the basal secretion of SPAP in the high expressing CHO-β_2/4 in a manner consistent with its inverse agonist properties (Samama *et al*., 1994; Figure 5a). In contrast, propranolol (1 μM) elicited a small, but significant ($P<0.05$), increase in basal SPAP release in the absence of agonist addition.

Salmeterol-stimulated gene expression

Salmeterol induced a similar maximal SPAP secretion to that obtained by isoprenaline in both cell lines (Figure 6; Table 3). This was in contrast to the clear difference in maximal responses (relative to isoprenaline) obtained from measurement of cyclic AMP accumulation (Table 3). Salmeterol was also more potent as a stimulant of SPAP secretion than it was of cyclic AMP accumulation (Table 3), although, the EC_{50} values for salmeterol-induced SPAP secretion were two orders

Table 2 Concentration response parameters for isoprenaline-stimulated cyclic AMP production and SPAP secretion

	Basal	E_{max}	$-Log\ EC_{50}$	n
(a) [^{3}H]-cyclic AMP (% conversion)				
CHO-β_2/4	3.8$\pm$1.4	9.9$\pm$2.7	8.6$\pm$0.2	4
CHO-β_2/6	0.6$\pm$0.2	4.3$\pm$1.3	7.1$\pm$0.2	4
(b) SPAP secretion (mU ml^{-1}; 24 h)				
CHO-β_2/4	0.041$\pm$0.011	0.132$\pm$0.023	8.7$\pm$0.2	8
CHO-β_2/6	0.037$\pm$0.004	0.058$\pm$0.007	7.3$\pm$0.4	4
(c) SPAP secretion (mU ml^{-1}; 5–6 h)				
CHO-β_2/4	0.005$\pm$0.001	0.014$\pm$0.001	9.3$\pm$0.3	9
CHO-β_2/6	0.009$\pm$0.002	0.017$\pm$0.003	7.2$\pm$0.3	4

Cyclic AMP accumulation and SPAP secretion was measured as described under Methods. Values represent means$\pm$s.e.mean of *n* separtate experiments. Concentration-response curves to isoprenaline were constructed in each experiment using triplicate determinants at each agonist concentration. The basal response represents that measured in response to addition of agonist vehicle. Concentration-response data in each individual experiment were fitted to a four-parameter logistic equation as described under Methods to provide fitted values for E_{max} and $-\log EC_{50}$.

Table 3 Concentration response parameters for salmeterol-stimulated cyclic AMP accumulation and SPAP secretion in CHO-K1 cells transfected with the human β_2-adrenoceptor

	Basal	E_{max}	*Isoprenaline* (1 μM)	E_{max} *(% of isop)*	$-Log\ EC_{50}$	n
(a) [^{3}H]-cyclic AMP (% conversion)						
CHO-β_2/4	2.71±0.51	6.41±0.89	6.28±0.77	104.34±12.66	8.89±0.47	5
CHO-β_2/6	0.22±0.03	0.45±0.04	1.29±0.21	22.42±5.32*	9.31±0.59	4
(B) SPAP secretion (mU ml^{-1}, 5–6 h)						
CHO-β_2/4	0.007±0.002	0.017±0.002	0.017±0.001	114.1±9.7	12.29±0.44	16
CHO-β_2/6	0.008±0.001	0.014±0.002	0.012±0.002	107.5±12.2	10.21±0.63	7

Cyclic AMP (cAMP) accumulation and SPAP secretion was measured as described under Methods. Values represent means±s.e.mean of n separate experiments. Concentration-response curves to salmeterol were constructed in each experiment using triplicate determinations at each agonist concentration. The basal response represents that measured in response to addition of agonist vehicle. Concentration-response data in each individual experiment were fitted to a four-parameter logistic equation as described under Methods to provide fitted values for E_{max} and $-\log EC_{50}$. In each experiment the response to 1 μM isoprenaline was also measured. (*) Significantly lower than the response to 1 μM isoprenaline ($P<0.05$; paired Student's t-test).

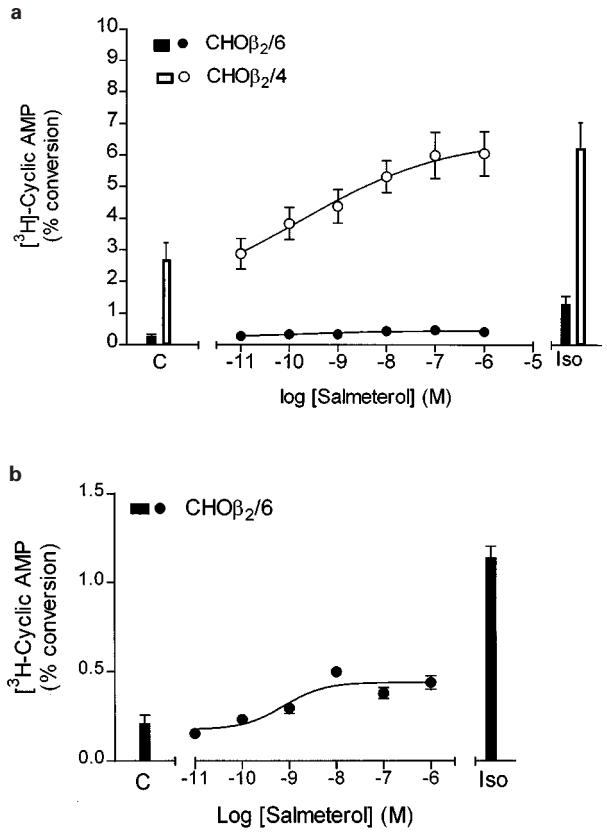

Figure 3 (a) Concentration-response curves for salmeterol-stimulated [^{3}H]-cyclic AMP accumulation in CHO-β_2/6 and CHO-β_2/4 cells. Values represent means±s.e.mean of triplicated determinations in 4 (CHO-β_2/6) and 5 (CHO-β_2/4) separate experiments. The response to 1 μM isoprenaline (Iso) was measured in each experiment (filled bar, CHO-β_2/6 cells; open bar, CHO-β_2/4 cells). The bars marked C show the basal [^{3}H]-cyclic AMP accumulation in the two cell lines (filled bar, CHO-β_2/6 cells; open bar, CHO-β_2/4 cells). (b) Concentration-response curve for salmeterol-stimulated [^{3}H]-cyclic AMP accumulation in CHO-β_2/6 cells. Data represent means± s.e.mean of triplicated determinations obtained in a single representative experiment. Similar data were obtained in three other experiments. The bars marked C and Iso show the basal and isoprenaline-stimulated (1 μM) [^{3}H]-cyclic AMP accumulation in the CHO-β_2/6 cells, respectively.

of magnitude lower in CHO-β_2/4 cells than in CHO-β_2/6 cells (Figure 6; Table 3).

The response to salmeterol was antagonized by ICI 118551 (Figure 7) in CHO-β_2/4 cells, leading to a marked shift to the right of the EC_{50} value for salmeterol by over two orders of magnitude. This effect of ICI 118551 was paralleled by a significant reduction of the salmeterol response to below basal levels, consistent with its inverse agonist properties (Figure 7).

Influence of serum on responses to salmeterol and isoprenaline

In the experiments described so far, the incubation medium in which SPAP secretion was measured contained 10% FCS. In order to evaluate the influence of this mitogenic agent on the concentration-response characteristics of the transcriptional effects of β_2-adrenoceptor agonists, we have conducted parallel experiments in cells which had been serum-starved for 24 h prior to agonist addition. Figure 8 compares data obtained with isoprenaline in serum-free and serum-containing medium. It is clear that the presence of 10% FCS alone can produce a direct stimulation of SPAP production over a 24 h period in the higher expressing CHO-β_2/4 cells (Figure 8a). However, it is striking that the presence of serum leads to a *circa* 10 fold increase in potency of isoprenaline (Figure 8a; Table 4). This effect of serum is still evident in cells which had been serum-starved for 24 h and only been exposed to serum for the 5 h incubation period with agonist (Figure 8b; Table 4). It is notable, however, under these latter conditions that the presence of serum has no direct effect alone (Figure 8b).

A similar 10 fold leftward shift in the position of the agonist concentration-response curve was obtained in the presence of 10% FCS with salmeterol in CHO-β_2/4 (Figure 9; Table 4). However, in the lower expressing CHO-β_2/6 cells the small shifts obtained with both isoprenaline and salmeterol in the presence of serum were not significant (Table 4).

Discussion

Isoprenaline-stimulated cyclic AMP in CHO-β_2/4 cells, which have a 6 fold higher expression of human β_2-adrenoceptors than CHO-β_2/6 cells, exhibited a larger maximal ^{3}H-cyclic AMP accumulation than in the latter cells. The location parameter (EC_{50}) of the agonist concentration-response curve in CHO-β_2/4 cells was also to the left (30 fold) of that for CHO-β_2/6 cells. These data confirm previous observations of the influence of β_2-adrenoceptor number on agonist concentration-response relationships (MacEwan *et al*., 1995; Bouvier *et al*., 1988; Whaley *et al*., 1994), and are indicative of the increased signal amplification, and consequent saturation of some of the intracellular signalling processes, resulting from the increased β_2-adrenoceptor density (Leff, 1995; Stephenson, 1956; Black & Leff, 1983). This suggests that amplification

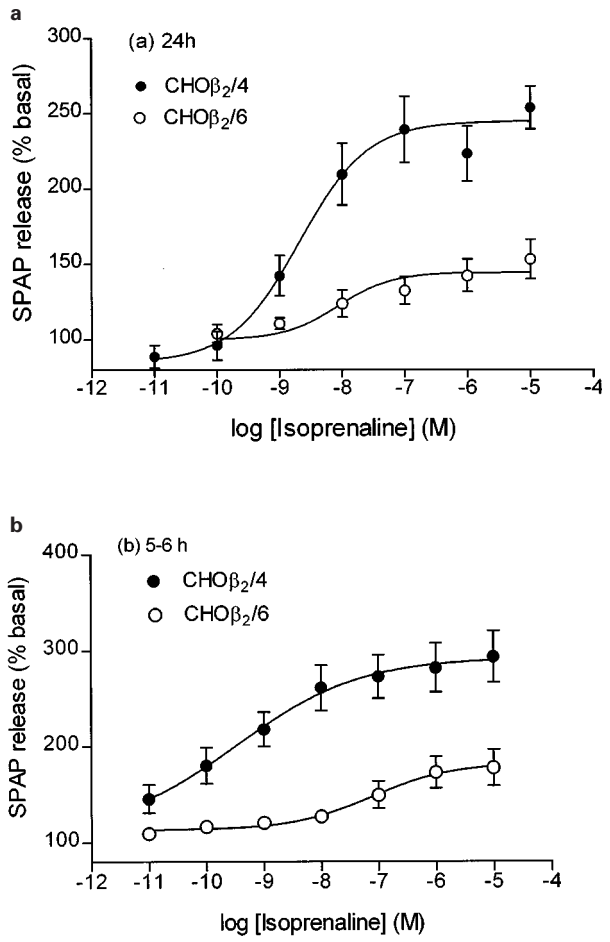

Figure 4 Stimulation of secreted placental alkaline phosphatase (SPAP) release from CHO-β_2/6 and CHO-β_2/4 cells in response to isoprenaline. Measurements were made (a) after 24 h or (b) between 5 and 6 h following isoprenaline administration. Data represent means ± s.e.mean of triplicate determinations obtained in 4 (CHO-β_2/6 cells, panels a and b), 8 (CHO-β_2/4 cells, (a)) or 11 (CHO-β_2/4 cells (b)) separate experiments. Values are expressed as a percentage of the basal SPAP release at each time point (basal = 100%).

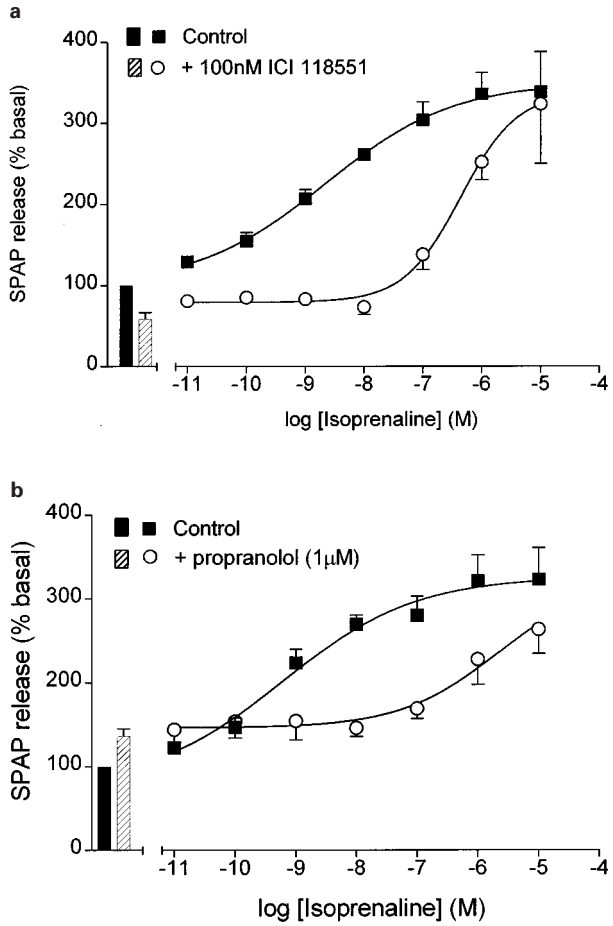

Figure 5 Effect of β-adrenoceptor-antagonists on isoprenaline-stimulated SPAP release (5 h) from CHO-β_2/4 cells. (a) Data obtained in the absence or presence of the β_2-selective adrenoceptor antagonist ICI 118551 (100 nM). (b) Data obtained in the absence or presence of the non-selective β-adrenoceptor antagonist propranolol (1 μM). Values are expressed as a percentage of the basal SPAP release at each time point (basal = 100%). Data represent means ± s.e.mean from triplicate determination in three (a) or four (b) separate experiments.

occurs between the binding of isoprenaline and the measured cyclic AMP response, and is consistent with recent observations that adenylyl cyclase may be the limiting step in β_2-agonist-stimulated cyclic AMP production (MacEwan *et al.*, 1996). The lower efficacy agonist salmeterol, which has been shown to exhibit partial agonist properties in a number of cells types (Coleman *et al.*, 1996; Clark *et al.*, 1996; McCrea & Hill, 1993, 1996), exhibited a lower intrinsic efficacy than isoprenaline in CHO-β_2/6 cells but not in CHO-β_2/4 cells (Table 3). Since the EC_{50} values for salmeterol were similar in the two cell lines, these data suggest that the receptor level in CHO-β_2/4 cells is only just sufficient for salmeterol to maximally activate adenylyl in these cells.

Another consequence of the higher β_2-adrenoceptor expression in CHO-β_2/4 cells is the appearance of agonist-independent receptor activation (Lefkowitz *et al.*, 1993; Milligan *et al.*, 1995). Thus, CHO-β_2/4 cells exhibited a higher basal accumulation of ^{3}H-cyclic AMP (relative to CHO-β_2/6 cells) that was reduced by application of a β_2-adrenoceptor antagonist (ICI 118551) with inverse agonist activity (Samama *et al.*, 1994). Constitutive β_2-adrenoceptor activity has been demonstrated previously following site directed mutagenesis of the β_2-adrenoceptor (Samama *et al.*, 1994) or following overexpression of the β_2-adrenoceptor in cultured cells

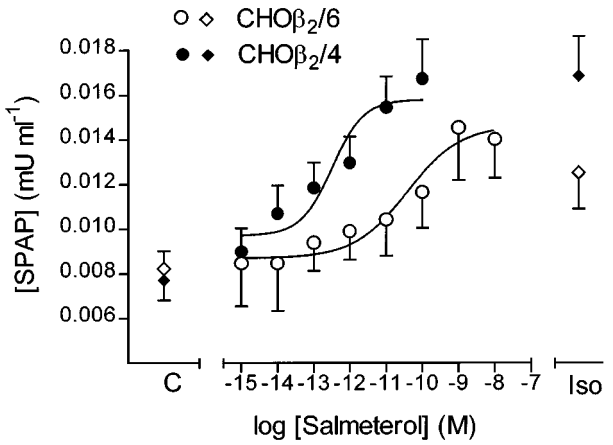

Figure 6 A compaison of concentration-response curves for salmeterol-stimulated SPAP release from CHO-β_2/4 and CHO-β_2/6 cells. Data represent means ± s.e.mean from triplicate determination in 16 (CHO-β_2/4) or seven (CHO-β_2/6) separate experiments. The response to 1 μM isoprenaline (Iso) was measured in each. Basal levels of SPAP secretion are shown at C. Agonist incubation periods were 5 h in all cases.

(Chidiac *et al*., 1994; Samama *et al*., 1994; Adie & Milligan, 1994) or transgenic mice (Milano *et al*., 1994).

Both isoprenaline and salmeterol were able to stimulate gene transcription in these cells *via* activation of a cyclic AMP response element controlling the production of the secreted reporter protein SPAP. Thus, both agents produced a reliable and reproducible response after an incubation period of 5 h. This length of incubation has been shown to be required, in studies of other CRE-based constructs which used either luciferase or chloramphenicol acetyltransferase as the reporter protein, to allow sufficient time for the accumulation of reporter gene protein (Guardiola-Diaz *et al*., 1994; Migeon *et al*., 1995; Pepperl & Regan, 1993). A comparison of the concentration-response parameters for isoprenaline-stimulated SPAP secretion (measured after 5 or 24 h, in the presence of serum) and cyclic AMP accumulation in CHO-β_2/6 cells (Table 2) showed very similar EC_{50} values. A similar observation was made in CHO-β_2/4 cells for cyclic AMP accumulation (after 10 min) and SPAP secretion after 24 h, although the SPAP secretion at 5–6 h was slightly more sensitive to isoprenaline ($-\log EC_{50}$ 9.3) than that at 24 h ($-\log EC_{50}$ 8.7). However, the EC_{50} value for isoprenaline-stimulated SPAP secretion ($-\log EC_{50}$ 7.2), obtained under serum free conditions (similar to the conditions in which cyclic AMP measurements were made; Table 4), was more than an order of magnitude less sensitive (24 fold) than the cyclic AMP response in CHO-β_2/4 cells ($-\log EC_{50}$ 8.6). In the lower expressing CHO-β_2/6 cells a smaller decrease in sensitivity of the SPAP response was observed with isoprenaline (5 fold).

In marked contrast, however, the stimulation of gene transcription was much more sensitive to salmeterol (measured

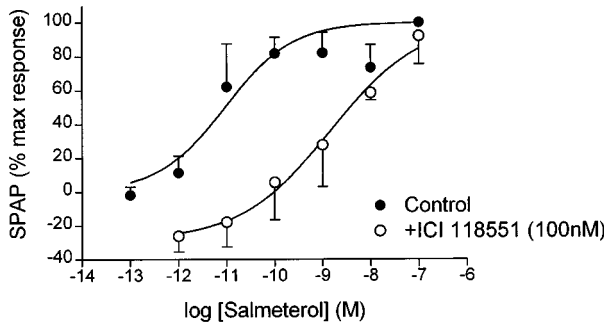

Figure 7 Effect of the β_2-adrenoceptor-selective antagonist ICI 118551 on salmeterol-stimulated (5 h) SPAP release from CHO-β_2/4 cells. Data were obtained in the absence or presence of the β_2-selective adrenoceptor antagonist ICI 118551 (100 nM). Values are expressed as a percentage of the response to 100 nM salmeterol in the absence of ICI 118551 obtained in each experiment (after subtraction of basal levels, obtained in the absence of ICI 118551). Data represent means ± s.e.mean from triplicate determination in three separate experiments.

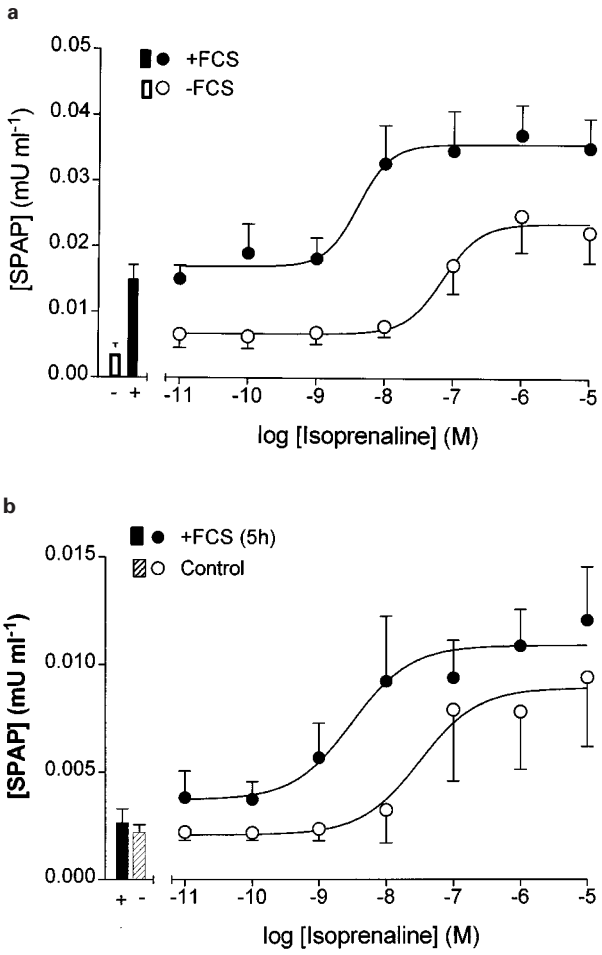

Figure 8 Influence of fetal calf serum (FCS) on the SPAP response to isoprenaline in CHO-β_2/4 cells. (a) Comparison of responses obtained in cells which had been serum starved for 24 h prior to assay in the continued absence of FCS, and cells which were incubated in medium containing 10% FCS throughout. (b) Effect of serum readdition. Cells were serum starved for 24 h prior to incubation with isoprenaline (5 h) in the presence or absence of 10% FCS. Values represent means ± s.e.mean of triplicate determination in (a) four and (b) three separate experiments. The basal release of SPAP obtained in the presence (+) and absence (−) of 10% FCS are shown by the bars.

Table 4 Influence of FCS on the concentration response parameters for β-agonist-stimulated SPAP secretion in CHO-K1 cells

	$-log\ EC_{50}$ (+serum)	$-log\ EC_{50}$ (−serum)
CHO4		
Isoprenaline	8.45 ± 0.19 (n = 4)*	7.22 ± 0.16 (n = 4)
Isoprenaline (serum 5 h#)	8.89 ± 0.56 (n = 3)*	7.42 ± 0.24 (n = 3)
Salmeterol	11.50 ± 0.20 (n = 3)*	10.60 ± 0.10 (n = 3)
CHO6		
Isoprenaline	6.78 ± 0.35 (n = 5)	6.39 ± 0.19 (n = 5)
Salmeterol	10.68 ± 0.25 (n = 3)	9.99 ± 0.30 (n = 3)

SPAP secretion was measured as described under Methods. Values represent means ± s.e.mean of n separate experiments. Concentration-response curves to isoprenaline and salmeterol (5 h incubation) were constructed in each experiment using triplicate determinations at each agonist concentration. Measurements were made in cells which had been serum starved for 24 h prior to assay in the absence of FCS (−serum) or in cells in which 10% FCS was present throughout (+serum). In some experiments, 10% FCS (serum 5 h#) was only present during the 5 h incubation with isoprenaline. Concentration-response data in each individual experiment were fitted to a four-parameter logistic equation as described under Methods to provide fitted values for $-\log EC_{50}$. (*) $P<0.05$ compared to parameters obtained in the absence of serum.

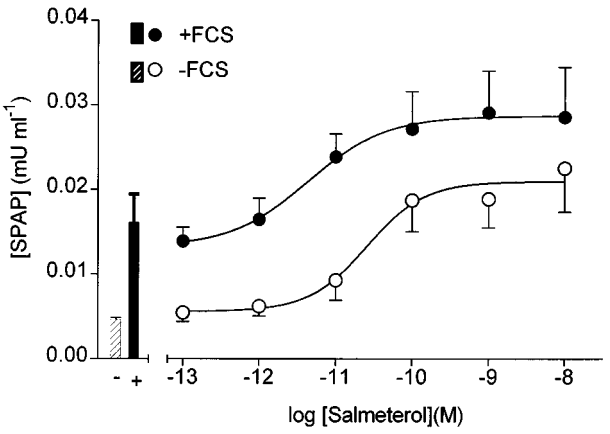

Figure 9 Influence of fetal calf serum (FCS) on the SPAP response to salmeterol (5 h) in CHO-β_2/4 cells. Cells were either serum starved for 24 h prior to assay in the continued absence of FCS or incubated in medium containing 10% FCS throughout. Values represent means ± s.e.mean of triplicate determination in three separate experiments. The basal release of SPAP obtained in the presence (+) and absence (−) of 10% FCS are shown by the bars.

in the presence or absence of FCS). Thus, in CHO-β_2/4 cells, the EC_{50} for SPAP secretion induced by salmeterol (under serum-free conditions) was 50 fold lower than the value for cyclic AMP accumulation, whereas in the presence of serum the SPAP response was some three orders of magnitude more sensitive ($EC_{50(cAMP)}/EC_{50(SPAP)} = 2511$; Table 3). In the lower expressing CHO-β_2/6 cells, however, the $EC_{50(cAMP)}/EC_{50(SPAP)}$ ratio was only 5 fold. Furthermore, in both cell lines salmeterol produced the same maximimal response as that obtained with isoprenaline. These data suggest that there is a substantial gain in efficiency occurring within the intracellular signal cascade from the initial agonist-receptor interaction (with salmeterol) at the plasma membrane and the stimulation of gene transcription in the nucleus. Consistent with this observation is the fact that a phosphodiesterase inhibitor (rolipram) was required to visualize the cyclic AMP responses in these cells to β-agonists, but not their effects on gene transcription.

The marked difference between isoprenaline and salmeterol in their ability to stimulate gene transcription under serum-free conditions is striking. In CHO-β_2/4 cells, salmeterol was able to maximally activate gene transcription at concentrations that had little effect on cyclic AMP accumulation (even in the presence of rolipram). In contrast, isoprenaline (a more efficacious agonist) required concentrations of agonist 24 fold higher, than required to increase cyclic AMP accumulation, to significantly alter gene transcription *via* CRE response elements. This is, at first sight, surprising since gene transcription *via* CRE response elements should parallel changes in cyclic AMP production. However, it should be emphasised that cyclic AMP accumulation was measured acutely (10 min), whereas the rate of SPAP secretion was monitored 6 h after the initial addition of agonist. It is likely that transcriptional responses to β_2-agonists are dependent upon both the rate of cyclic AMP generation and the duration of the CRE-stimulus. If both agonists retained their activity over the full transcriptional stimulation period, then they should have a similar response profile. The data obtained in the present study suggest that they do not.

These data suggest that the expected large leftward-shift in the concentration-response curve for isoprenaline-stimulated SPAP secretion (relative to the corresponding cyclic AMP curve) may have been offset by both ongoing receptor desensitization (during the longer incubation period required for SPAP measurements) and as a consequence of spontaneous oxidation and/or metabolism of isoprenaline. Furthermore, it is likely that some desensitization of the cyclic AMP response to isoprenaline will have occurred even during the 10 min incubation period with the higher concentrations of agonist. Thus, the EC_{50} values for the stimulation of the initial rate of cyclic AMP production by isoprenaline might be further left-shifted from the observed SPAP concentration-response curve. The much longer duration of action of salmeterol at the β_2-adrenoceptor (relative to isoprenaline; Coleman *et al*., 1996; Clark *et al*., 1996; McCrea & Hill, 1996; Green *et al*., 1996) may therefore be advantageous in responses, such as gene transcription, which take many hours to develop. Furthermore, previous studies have suggested that the response to salmeterol is less sensitive to desensitization than isoprenaline (Coleman *et al*., 1996; Clark *et al*., 1996). This may be due to the lower intrinsic activity of salmeterol (Clark *et al*., 1996; Inglese *et al*., 1993; Yuan *et al*., 1994; Benovic *et al*., 1988). Further studies of the effect of agonist incubation time, with chemically stable β_2-agonists (both high and low efficacy), on gene transcription (measured after 6 h) should provide further information on this point.

It was notable that the basal SPAP secretion in CHO-β_2/4 cells (in the absence of agonist) was reduced following incubation with the inverse agonist ICI 118551 (Figure 5a), indicating that constitutive β-adrenoceptor activity was manifest at the level of gene transcription. Similarly, ICI 118551 shifted isoprenaline concentration-response curves for SPAP secretion in these cells to higher agonist concentrations in the manner predicted by the two-site model for the presence of constitutive basal receptor activation (Leff, 1995). A similar antagonism of the isoprenaline response was observed with propranolol, although this β-antagonist produced a small direct stimulation of SPAP release on its own (Figure 5b).

The presence of serum in the culture medium immediately prior to, and during, the period of agonist stimulation required for receptor-induced SPAP secretion produced two different effects on CRE-mediated gene transcription. The marked increase in basal SPAP secretion observed in cells continually exposed to 10% FCS, compared to that in serum-starved cells (e.g. Figure 8a) is likely to be due to general pleotrophic effects of this mitogen, since it was not observed when serum was only present during the β_2-agonist incubation period (Figure 8b). However, the effect that was observed under both of these conditions was the shift of β_2-agonist concentration-response curves to lower agonist concentrations. These data suggest that there are amplifying interactions occurring between the different intracellular signalling pathways activated by serum and β_2-agonists in these cells leading to activation of CRE-mediated transcription. The effects of β-adrenoceptor stimulation on the CRE-sensitive reporter gene are most likely to be a consequence of the phosphorylation *via* protein kinase A of serine-133 within the cyclic AMP response element-binding protein CREB enabling it to interact with the adaptor molecule CBP (CREB-binding protein; Lalli & Sassone-Corsi, 1994; Swope *et al*., 1996; Nordheim, 1994). Evidence is accumulating that CREB can also be phosphorylated on serine-133 by growth factors *via* the RAS/MAP kinase pathway and the subsequent activation of the CREB kinase Rsk-2 (Xing *et al*., 1996; Trivier *et al*., 1996). CREB phosphorylation thus provides a point of convergence between the pathways activated by serum and β-agonists. Recent studies have suggested that several protein kinase A -mediated events are required for CREB-mediated transcription, in addition to serine-133 phosphorylation (Swope *et al*., 1996;

Brindle *et al*., 1995). For example, in T cells serine-133 phosphorylation induced by the adenylyl cyclase activator forskolin is sufficient to activate a CRE-based reporter gene, while a similar level of serine-133 phosphorylation induced by a monoclonal antibody to the T cell receptor is not (Brindle *et al*., 1995). Thus, protein kinase A stimulated phosphorylation of other transcriptional domains of CBP (Swope *et al*., 1996) in addition to serine-133 phosphorylation of CREB, may provide a mechanism to explain the observed synergy between growth factors (in FCS) and β-agonists on CRE-mediated transcription in the present study.

In summary, it is clear that salmeterol can produce a signinificant stimulation of gene transcription at very low agonist concentrations. The low efficacy of this β_2-adenoceptor agonist (and consequent reduced susceptability to desensitization) together with its long duration of action favours this response over more efficacious but much shorter-lived agonists such as isoprenaline. Furthermore, the observation that serum-derived growth factors can augment the action of β_2-agonists on CRE-mediated reporter gene expression provides the potential for the sensitivity of PKA transcriptional pathway to be dependent upon the degree of activation of coincident intracellular signalling pathways. This may be important when β_2-agonists are used to treat inflammatory disease where a wide range of inflammatory mediators may be present.

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