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Influence of Agonist Efficacy and Receptor Phosphorylation on Antagonist Affinity Measurements: Differences between Second Messenger and Reporter Gene Responses

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ABSTRACT

The ability of an antagonist to bind to a receptor is an innate property of that ligand-receptor chemical interaction. Provided no change in the antagonist or receptor chemical nature occurs, this affinity should remain constant for a given antagonist-receptor interaction, regardless of the agonists used. This fundamental assumption underpins the classification of receptors. Here, measurements of β_2-adrenoceptor-mediated cAMP accumulation and cAMP response-element (CRE)-mediated reporter-gene transcription revealed differences in antagonist affinity that depended upon agonist incubation time and the efficacy of the competing agonist. In cAMP accumulation studies (10-min agonist incubation), antagonist affinities were the same regardless of the agonist used. The CRE-reporter gene assay (5 h of incubation) antagonist affinities were 10-fold lower in the presence of isoprenaline and adrenaline than when salbutamol or terbutaline were present (e.g., log K_D propranolol -8.65 ± 0.08, $n = 22$, and -9.68 ± 0.07, $n = 17$, for isoprenaline and salbutamol-induced responses, respectively). Isoprenaline and adrenaline were more efficacious in functional studies, and their ability to internalize GFP-tagged human β_2-adrenoceptors. Longer-term cAMP studies also showed significant differences in K_D values moving toward that seen with gene transcription. Agonist-dependent differences in antagonist affinity were reduced for reporter-gene responses when a phosphorylation-deficient mutant of the β_2-adrenoceptor was used. This study suggests that high-efficacy agonists induce a chemical modification in β_2-adrenoceptors (via phosphorylation) that reduces antagonist affinities. Because reporter-gene assays are used for high-throughput screening in drug discovery, less efficacious or partial agonists may be more reliable than highly efficacious agonists when reporter-gene techniques are used to estimate antagonist affinity.

The ability of an agonist to stimulate a response via a receptor depends on two factors: 1) its ability to bind to the receptor (affinity) and 2) its ability to induce a response (efficacy) (Kenakin and Onaran, 2002). However, the receptor activation process itself often induces feedback mechanisms that act to "turn off" the activated receptor and thus "reset" the receptors so that they are able to respond to future stimulation (Clark et al., 1999; Tsao et al., 2001). In the case of the human β_2-adrenoceptor, coupling to Gs-proteins leads to activation of adenylyl cyclase, cAMP formation, and a subsequent increase in the activity of protein kinase A (PKA) (Kobilka, 1992). PKA then phosphorylates intracellular targets including cAMP response element binding protein and the β_2-adrenoceptor itself (serines at positions 261 and 262 on the third intracellular loop and 345 and 346 on the C terminus; Yuan et al., 1994; Clark et al., 1999; Seibold et al., 2000). This leads to rapid desensitization of β_2-adrenoceptor-induced stimulation of adenylyl cylcase (Yuan et al., 1994). However, if enough β_2-adrenoceptors are activated by a sufficiently efficacious agonist (e.g., isoprenaline), G-protein-coupled receptor kinases GRK 2 and 3 (also known as β-ARK 1 and 2) are recruited from the cytoplasm. These phosphorylate the C terminus of the receptor (serines at positions 355, 356, 364; Fredericks et al., 1996; Clark et al., 1999; Seibold et al., 2000) and enable β-arrestin to bind to the β_2-adrenoceptor. β-Arrestin acts as an adapter protein and recruits the receptor to clathrin-coated pits, which cause its subsequent internalization (Krupnick and Benovic, 1998; Clark et al., 1999; Kohout and Lefkowitz, 2003).

The efficacy of an agonist is closely related to its ability to phosphorylate and internalize the receptor (January et al., 1997, Clark et al., 1999). It seems that PKA-mediated phos-

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ABBREVIATIONS: PKA, protein kinase A; CRE, cAMP response element; SPAP, secreted placental alkaline phosphatase; CHO, Chinese hamster ovary; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F12; IBMX, 3-isobutyl-1-methylxanthine; BAAM, bromoacetyl alprenolol methane; ICI 118551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; GRK, G-protein-coupled receptor kinase; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride.

phorylation of the β_2-adrenoceptor occurs with relatively low levels of cAMP (i.e., from low receptor occupancy of an efficacious agonist or from a higher occupancy of a partial agonist) and causes partial uncoupling of the receptor from Gs-proteins (40–60%; Yuan et al., 1994). For GRK (β-ARK) phosphorylation to occur, however, high receptor occupancy from an efficacious agonist is required, which rapidly inactivates the receptor by phosphorylation and internalization (Clark et al., 1999).

Neutral antagonists have little or no efficacy of their own and their affinity for a particular receptor can be calculated from its ability to block agonist responses (Arunlakshana and Schild, 1959). The ability of an antagonist to bind to a given receptor is an innate property of the chemical composition of the ligand-receptor interaction (Kenakin et al., 1995). Provided there is no change in the chemical nature of the antagonist or receptor, this affinity should remain constant for a given receptor-antagonist interaction, regardless of which agonists are present or what downstream signaling events are monitored. Thus an antagonist's ability to bind to a given receptor and block an agonist response should be constant no matter whether an immediate event (e.g., second messenger changes) or further downstream event (e.g., gene transcription) is being measured. Consequently, differences in the antagonist affinity has long underpinned the characterization of receptors and their subtypes and drug discovery programs (Arunlakshana and Schild, 1959; Black et al., 1965, 1972) and more recently the characterization of secondary binding sites (Kenakin and Boselli, 1989; Konkar et al., 2000).

In recent years, reporter gene techniques have been used in drug discovery as a readout of the cell surface G-protein-coupled receptor agonist/antagonist interactions (Rees et al., 1999). Here, a DNA sequence is transfected into the cells containing a promotor sequence for the signaling cascade of interest [e.g., a cyclic AMP response element (CRE)] upstream of a sequence that encodes for a unique and easily measured novel protein product [e.g., luciferase, secreted placental alkaline phosphatase (SPAP)] (Hill et al., 2001). However, these techniques normally require several hours from addition of agonists until the reporter gene product can be measured (Rees et al., 1999; Hill et al., 2001; Baker et al., 2003). During this time, however, it is likely that the chemical nature of the receptor will have been modified by the processes of phosphorylation, internalization, and recycling.

The aim of this study, therefore, was to determine whether a long-term reporter gene assay gives the same affinity constants for antagonists as traditional, short-term, upstream second messenger readouts. We have also investigated whether any differences obtained are dependent upon the efficacy of the agonist used to stimulate responses.

Materials and Methods

Materials. Cell culture reagents were from Sigma Chemicals (Poole, Dorset, UK) except fetal calf serum, which was from PAA laboratories (Teddington, Middlesex, UK). [^{3}H]adenine and [^{14}C]cAMP were obtained from Amersham Biosciences (Buckinghamshire, UK). All other reagents were supplied by Sigma Chemicals.

Cell Culture. Experiments were performed using CHO cells stably expressing both the human β_2-adrenoceptor and an SPAP reporter gene under the transcriptional control of a six-CRE promoter (CHO-β_2 cells; 300–400 fmol/mg protein; McDonnell et al., 1998). A stable line of CHO cells expressing a GFP-tagged human β_2-adrenoceptor (CHO-β_2-GFP) was used in confocal studies (Baker et al., 2002a). A stable CHO cell line was made by transfection with the CRE-SPAP reporter and a mutant β_2-adrenoceptor possessing mutated β-ARK and PKA phosphorylation sites (DNA was a gift from Prof. R. B. Clarke, University of Texas, Houston, TX) (Seibold et al., 2000) using LipofectAMINE and Opti-MEM according to the manufacturer's instructions (CHO-$\beta_2$mut-SPAP). Transfected cells were selected using resistance to G-418 (1 mg/ml; for $\beta_2$mut) and hygromycin (200 μg/ml; for CRE-SPAP). A single clone was isolated by dilution cloning. Untransfected cells (CHO-K1) and a stable line transfected only with the CRE-SPAP reporter (CHO-SPAP) were used as controls where stated. All CHO cell lines were grown at 37°C in Dulbecco's modified Eagle's medium/Ham's nutrient mix F12 (DMEM/F12) containing 10% fetal calf serum and 2 mM L-glutamine in a humidified 5% CO_2/95% air atmosphere.

CRE-Mediated Gene Transcription (SPAP). Cells were grown to confluence in 24-well plates then serum-starved for 24 h before experimentation in DMEM/F12 containing 2 mM L-glutamine (serum-free media). On the day of experimentation, the media was replaced with 1 ml of fresh serum-free media. Where used, antagonists were added to this media and incubated for 30 min at 37°C in a humidified atmosphere of 5% CO_2/95% air. Agonists (in 10 μl, each condition in triplicate) were then added and incubated for 5 h in the same atmosphere. Media and drugs were then removed and replaced with 300 μl of fresh serum-free media and incubated for a further hour. Samples of media (20 μl) from each well were then transferred to 96-well plates and heated to 65°C for 30 min to destroy any endogenous alkaline phosphatases. Thus the rate of CRE gene transcription and SPAP secretion at 5 to 6 h was measured rather than the total accumulative SPAP secretion over 5 h. CRE-dependent SPAP reporter activity was quantified by following the color change caused by the hydrolysis of *p*-nitrophenol phosphate (Cullen and Malim, 1992). *p*-Nitrophenol phosphate (200 μl) in diethanolamine buffer was added to each sample and incubated at 37°C in air for 1 h. The plates were then read at 405 nm using a Dynatech Laboratories MRX plate reader, and the data was converted to SPAP concentration (milliunits per milliliter) as described previously (McDonnell et al., 1998).

Cyclic AMP Accumulation. Cells were grown to confluence in 24-well plates then prelabeled with [^{3}H]adenine (2 μCi/ml) for 2 h at 37°C in 1 ml/well Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4. The [^{3}H]adenine was removed, each well washed with 1 ml of Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4, then incubated for 30 min with 1 ml medium containing IBMX (1 mM). Any antagonists used were added at this stage and thus, as in the gene transcription assay, had a 30-min preincubation before the agonist addition. Agonists in 10 μl were then added and the cells were incubated for 10 min before the reaction was terminated by the addition of 50 μl of concentrated HCl. [^{3}H]Cyclic AMP was separated from other [^{3}H]adenine nucleotides by sequential Dowex and alumina chromatography, and each was column corrected for efficiency by comparison with [^{14}C]cAMP recovery as described previously (Donaldson et al., 1988).

Where BAAM (an irreversible β-antagonist, MacEwan et al., 1995) was used, it was added to the cells with the [^{3}H]adenine and thus had 2 h of incubation at 37°C. It was washed away with the removal of the [^{3}H]adenine and not replaced for the 30-min IBMX or 10-min agonist incubations. For the long-term cAMP measurements, the gene transcription assay was mimicked as far as possible; thus, experiments were performed in serum-free media and in the absence of IBMX. After the 2-h [^{3}H]adenine prelabeling, antagonists were added for 30 min followed by a 5-h agonist incubation. Total well cAMP accumulation was measured by adding 50 μl of concentrated HCl to the wells as described above. In other wells of the same experiment, intracellular and extracellular (secreted) cAMP were also measured separately. The extracellular media was removed

(and acidified) and assayed for cAMP. The remaining cells were then washed three times by the addition and removal of 1 ml of serum-free media before a further 1 ml of serum free media was added. The cells were then lysed by the addition of 50 μl of concentrated HCl to the well, and the intracellular cAMP was measured by assaying these well contents as described above.

Confocal Microscopy. Confocal microscopy was performed using a Zeiss LSM 510 laser scanning microscope (Argon laser, 488 nm line; 505 nm long-pass filter) with a Zeiss 40 $\times$ 1.3 numerical aperture oil immersion lens. CHO-β_2-GFP cells were grown on glass coverslips in 6 well plates containing 3 ml of DMEM/F12 media containing 10% fetal calf serum and 2 mM glutamine. The coverslips were transferred to a specially designed holder in a heated stage to form the base of a sealed chamber to which 1 ml of HEPES-buffered saline was added. The microscope objective and stage were maintained at 37°C throughout the experiments. Agonists (in 10 μl HEPES-buffered saline) were added for 30 min, and the cells were imaged in the continued presence of agonist (1024 $\times$ 1024 pixels; averaging at four frames).

Data Analysis. A maximal isoprenaline concentration (10 μM) was included in each separate experiment for both [^{3}H]cAMP accumulation and SPAP gene transcription to allow the other agonist responses to be expressed as a percentage of the isoprenaline maximum (except Fig. 1a, in which the isoprenaline concentration curve provides a measure of the isoprenaline maximum response). Sigmoidal agonist concentration-response curves (in the presence and absence of antagonists) were fitted to the following equation through computer-assisted nonlinear regression using the program Prism 2 as described previously (Hopkinson et al., 2000): Response = $(E_{max} \times 10^{log[A]}) / (10^{logEC_{50}} + 10^{log[A]})$, where E_{max} is the maximal response, [A] is the agonist concentration, and EC_{50} is the concentration of agonist that produces 50% of the maximal response.

Antagonist dissociation constants were assessed at fixed antagonist concentrations (assuming competitive antagonism) by observing the shift in the agonist concentration-response curve using the equation: $DR = 1 + [A]/K_D$, where DR (dose-ratio) is the ratio of the concentrations of agonist required to produce an identical response in the presence and absence of antagonist, [A] is the concentration of antagonist, and K_D is the antagonist dissociation constant.

Schild slopes (n) were obtained from linear regression of the Schild equation: $Log(DR - 1) = nlog[A] - logK_D$. All data are presented as mean $\pm$ S.E.M. The n in the text refers to the number of separate experiments.

Results

Differences in Antagonist K_D Values in CRE-Mediated Gene Transcription (SPAP) Measurements in CHO-β2-SPAP Cells. Isoprenaline stimulated an increase in CRE-mediated SPAP production that was 6.53 $\pm$ 0.37-fold higher than the basal response ($logEC_{50} = -8.11 \pm 0.09$, n = 40) (Fig. 1a, Table 1). Adrenaline, salbutamol, and terbutaline stimulated similar responses (Fig. 1, Table 1), showing that they all appear as full agonists of CRE-mediated gene transcription in this cell system. Increasing concentration of ICI 118551, a selective β_2-inverse agonist, inhibited the isoprenaline-induced response shifting the concentration-response curve in a parallel manner consistent with competi-

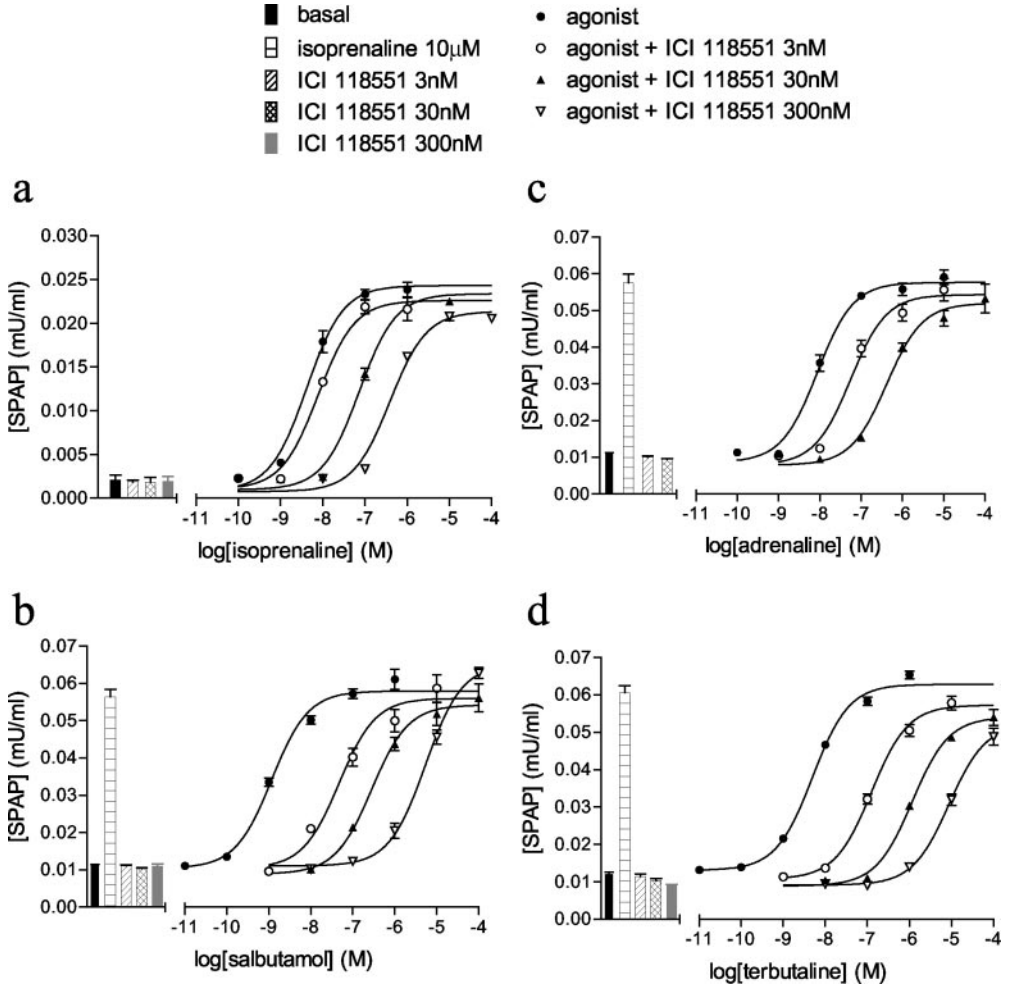

Fig. 1. CRE-mediated SPAP production in CHO-β_2 cells in response to isoprenaline (a), salbutamol (b), adrenaline (c) and terbutaline (d) in the absence and presence of 3, 30, and 300 nM ICI 118551. Bars represent basal SPAP production and that in response to 10 μM isoprenaline or 3, 30, or 300 ICI 118551 alone. The data points are mean $\pm$ S.E.M. (triplicate determinations) from a single experiment and these are representative of 31 (a), 26 (b), 8 (c), and 13 (d) separate determinations. The Schild slopes are 0.93 $\pm$ 0.07 (n = 3) (a), 0.96 $\pm$ 0.03 (n = 3) (b), insufficient curves for Schild plot (c) and 1.01 for this experiment (d); in the other experiments, only two concentrations of antagonist were used.

TABLE 1

$LogEC_{50}$ values for agonists at the level of cAMP accumulation and CRE-SPAP gene transcription

E_{max} values show the maximal response to each agonist expressed as a percentage of the maximal response to isoprenaline measured in the same experiment. Values are mean ± S.E.M. of n separate determinations.

	cAMP			CRE Gene Transcription (SPAP)		
	$LogEC_{50}$	E_{max}	n	$LogEC_{50}$	E_{max}	n
		%			%	
Isoprenaline	−8.75 ± 0.05	100	19	−8.11 ± 0.09	100	40
Adrenaline	−8.39 ± 0.05	93.2 ± 1.5	7	−7.62 ± 0.14	107.7 ± 2.9	8
Salbutamol	−7.45 ± 0.04	95.8 ± 2.2	21	−8.66 ± 0.05	97.0 ± 1.4	34
Terbutaline	−7.06 ± 0.05	100.6 ± 6.9	6	−8.33 ± 0.07	100.2 ± 1.8	15

tive antagonism to yield a log K_D value of −9.08 ± 0.07, n = 31 (Fig. 1a, Table 2). Responses to adrenaline were antagonized in a similar manner (log K_D for ICI 118551 = −9.03 ± 0.11, n = 8, Fig. 1c, Table 2). Responses to salbutamol and terbutaline were also antagonized by 3 to 300 nM ICI 118551; however, the log K_D values obtained (−9.97 ± 0.06, n = 26 and −9.92 ± 0.07, n = 13, respectively) were significantly different from those values obtained when isoprenaline and adrenaline were agonists ($p < 0.001$, one-way analysis of variance; Fig. 1, b and d; Table 2). Thus, the concentration of ICI 118551 required to antagonize the isoprenaline and adrenaline induced responses was 10 times that required to antagonize the salbutamol- and terbutaline-induced responses.

Propranolol gave a similar pattern of antagonism. The salbutamol and terbutaline-induced responses were antagonized by 3 to 300 nM propranolol in a competitive manner (Table 2); however, the isoprenaline and adrenaline responses again required 10-fold higher concentrations of the antagonist (Table 2). Atenolol also gave a similar pattern (Table 2). Thus, regardless of the antagonist used, in this assay requiring a 5-h incubation with agonist, 10-fold higher concentrations of antagonist were needed to antagonize the isoprenaline- and adrenaline-induced responses than for the salbutamol- and terbutaline-induced responses (Table 2). To further investigate this discrepancy in K_D values, cAMP accumulation assays were performed that involved only a 10-min agonist incubation.

Similar Antagonist K_D Values in [^{3}H]cAMP Accumulation Measurements in CHO-β_2-SPAP Cells. Isoprenaline stimulated an increase in [^{3}H]cAMP accumulation after a 10-min incubation that was 21.7 ± 2.9-fold over basal ($logEC_{50}$ = −8.75 ± 0.05, n = 19) (Table 1, Fig. 2a). Again, adrenaline, salbutamol, and terbutaline induced similar full agonist responses (Table 1). ICI 118551 antagonized the isoprenaline-induced [^{3}H]cAMP accumulation responses, to give a log K_D value of −9.43 ± 0.09, n = 15 (Fig. 2a). This was very similar to the values obtained when adrenaline, salbutamol, and terbutaline were used as the agonists (Fig. 2b, Table 2). Interestingly, when salbutamol and terbutaline were incubated with increasing concentrations of ICI 118551, a progressive decrease in the maximal response obtained was seen (Fig. 2b), suggesting that only a hemi-equilibrium was achieved with this slowly dissociating antagonist (Hopkinson et al., 2000). In view of this, apparent antagonist K_D values were only calculated from the first shift with the lowest concentration of antagonist.

These data obtained with salbutamol and terbutaline and ICI 118551 in the 10-min cAMP assays provide some evidence that an incomplete equilibration exists during this short incubation. The importance of equilibrium kinetics in the interaction of agonists and antagonists with G-protein-coupled receptors has been emphasized by Motulsky and Mahan (1984). To ensure that the differences in antagonist K_D (with different agonists) observed between the cAMP and CRE-SPAP assays are not caused by differences in the extent to which equilibration is achieved, experiments were undertaken with propranolol as the antagonist. This compound has been shown previously to equilibrate quickly with the β_2-adrenoceptor (Motulsky and Mahan (1984) ($t_{1/2}$ for dissociation = 0.69 min). When propranolol was used as the antagonist, the log K_D values obtained with isoprenaline, adrenaline, salbutamol, and terbutaline were again very similar, and this short-acting antagonist did not cause a reduction in the maximum responses (Fig. 3). Similar data were obtained using atenolol as the antagonist (Table 2). Full equilibrium therefore seems to have been reached (Table 2)

TABLE 2

log K_D values of antagonists obtained in the presence of four different agonists in the cAMP accumulation and CRE-SPAP gene transcription assays

Values are mean ± S.E.M of n separate determinations. There are no significant differences for each antagonist K_D values obtained with the different agonists in the cAMP accumulation experiments or between the K_D values obtained for the isoprenaline- and adrenaline-induced gene transcription responses for either ICI 118551 or propranolol as the antagonist. There were also no significant differences between the antagonist K_D values obtained when salbutamol and terbutaline were agonists in the gene transcription assay.

	Isoprenaline	n	Adrenaline	n	Salbutamol	n	Terbutaline	n
cAMP Accumulation								
ICI 118551	−9.43 ± 0.09	15	−9.54 ± 0.05	7	−9.32 ± 0.12	8	−9.41 ± 0.04	3
Propranolol	−9.40 ± 0.06	11	−9.32 ± 0.06	3	−9.52 ± 0.06	8	−9.32 ± 0.06	3
Atenolol	−5.82 ± 0.07	9	−5.77 ± 0.04	3	−5.87 ± 0.05	10	−5.70 ± 0.00	3
CRE-SPAP Gene Transcription								
ICI 118551	−9.08 ± 0.07	31	−9.03 ± 0.11	8	−9.97 ± 0.06*	26	−9.92 ± 0.07*	13
Propranolol	−8.65 ± 0.08	22	−8.65 ± 0.2	7	−9.68 ± 0.07*	17	−9.64 ± 0.05*	9
Atenolol	−5.27 ± 0.13	12	−4.77 ± 0.19†	5	−6.12 ± 0.04*	9	−6.24 ± 0.07*	7

* Significantly different ($p < 0.001$, one-way analysis of variance, Neuman-Keul's post hoc) from the values obtained with isoprenaline or adrenaline as the agonist.
† Significance of $p < 0.01$ compared with the isoprenaline-induced K_D value for atenolol.

with propranolol and atenolol within the 10-min agonist incubation.

To confirm the lower agonist efficacies of salbutamol and terbutaline, experiments were conducted using BAAM, an irreversible β-antagonist (MacEwan et al., 1995). BAAM itself stimulated a small agonist response (Fig. 4); however, the parent compound, alprenolol, has also been reported to stimulate small [^{3}H]cAMP accumulation responses (Baker et al., 2002b). The isoprenaline-induced [^{3}H]cAMP accumulation response was shifted to the right after 2-h preincubation with 100 nM and 1 μM BAAM but with little decrease from the maximum response (Fig. 4a, $n = 5$). The maximum salbutamol-induced responses however, were not only right-shifted but also markedly reduced by preincubation with 100 nM and 1 μM BAAM (Fig. 4b, $n = 5$), thus demonstrating the lower efficacy of salbutamol (compared with isoprenaline) at this receptor.

Confocal Imaging of CHO-β_2-GFP Cells. Under basal conditions, the GFP-tagged β_2-adrenoceptor in CHO-β_2-GFP cells was localized to the plasma membrane (150 cells imaged of 49 different wells; Fig. 5). After a 30-min incubation with isoprenaline (1 μM and above), the GFP-tagged receptor moved from the membrane into discrete lesions within the cytoplasm, where it remained for at least 1 h (47 cells in 20 wells; Fig. 5). Incubation with 10 μM adrenaline caused a similar intracellular movement of the GFP-tagged receptor (21 cells in 7 wells; Fig. 5). In contrast, incubation with salbutamol and terbutaline (up to concentrations of 100 μM) caused only slight, if any, internalization of the GFP-tagged β_2-receptor (44 cells in 13 wells for salbutamol and 38 cells in

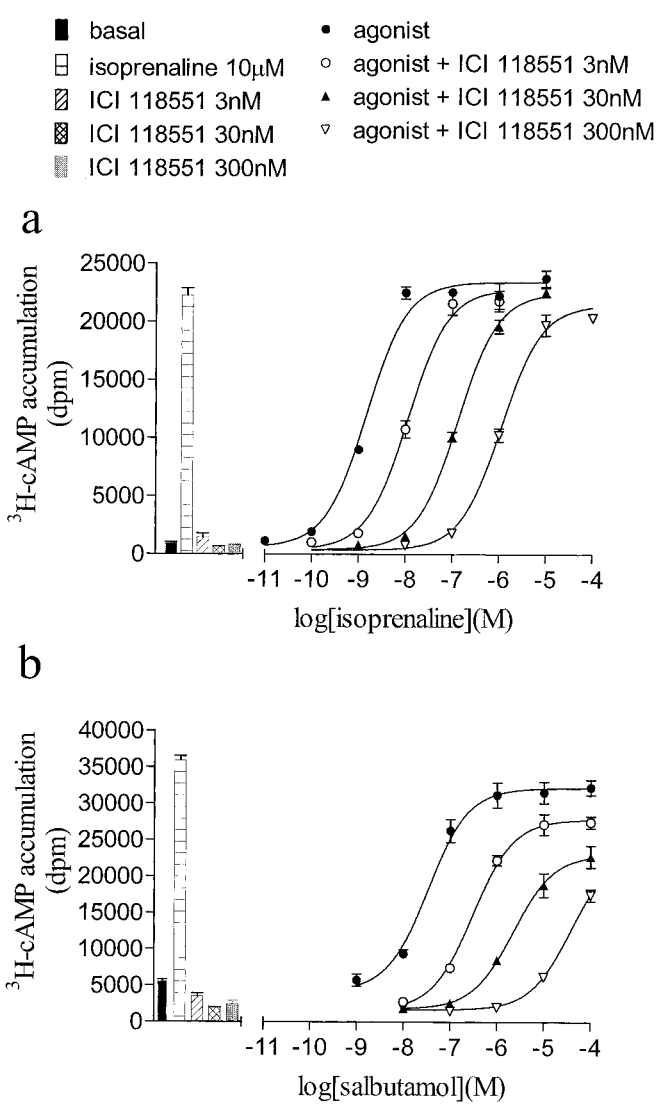

Fig. 2. [^{3}H]Cyclic AMP accumulation in CHO-β_2 cells in response to isoprenaline (a) and salbutamol (b) in the presence and absence of 3, 30, and 300 nM ICI 118551. Bars represent basal [^{3}H]cAMP accumulation and that in response to 10 μM isoprenaline or 3, 30, or 300 nM ICI 118551 alone. All data points are mean ± S.E.M. from triplicate determinations in a single experiment and these experiments are representative of 15 (a) and 3 (b) separate determinations. In those experiments that used three different antagonist concentrations, the Schild slopes for a were 1.08 ± 0.04 ($n = 3$), whereas b displays noncompetitive inhibition.

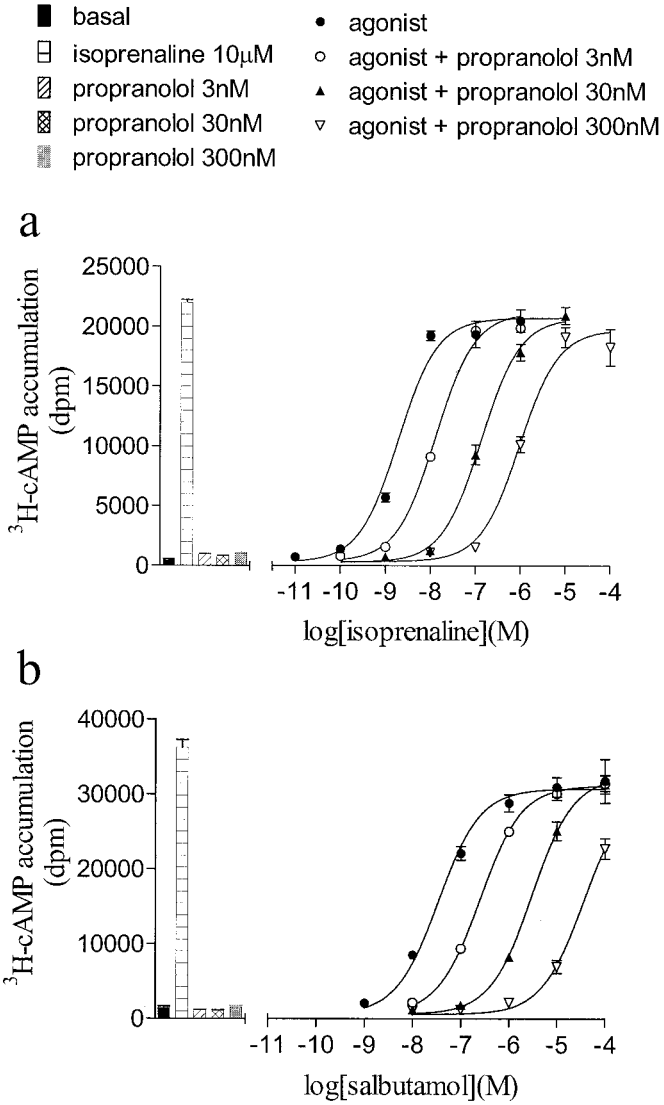

Fig. 3. [^{3}H]cyclic AMP accumulation in response to isoprenaline (a) and salbutamol (b) in the presence and absence of 3, 30, and 300 nM propranolol. Bars represent basal [^{3}H]cAMP accumulation and that in response to 10 μM isoprenaline or 3, 30, or 300 nM propranolol alone. All data points are mean ± S.E.M. (triplicate determinations) from a single experiment and these single experiments are representative of 11 (a) and 8 (b) separate determinations. The Schild slopes determined from experiments in which three different antagonist concentrations were used are 1.09 ± 0.04 ($n = 3$) (a) and 1.02 and 1.08 on two different occasions (b).

9 wells for terbutaline; Fig. 5). This suggests that these four agonists have different abilities to internalize the β_2-adrenoceptor.

Long-Term [^{3}H]cAMP Studies Reveal Differences in K_D Values. To evaluate the contribution of the agonist incubation time to the antagonist affinity measurements, [^{3}H]cAMP measurements were made in a manner as close to the conditions of the CRE-gene transcription assay as possible; thus, these experiments were performed in serum-free media in the absence of IBMX. After 5-h incubation with isoprenaline, the intracellular [^{3}H]cAMP accumulation was not detectable (Fig. 6) above baseline. Measurements of total (intracellular plus extracellular) [^{3}H]cAMP accumulation over 5 h were therefore performed. Under these conditions, isoprenaline stimulated an increase in 5 h of total accumulation of [^{3}H]cAMP that was 50.7 ± 7.4-fold higher than basal (logEC_{50} = −8.23 ± 0.05, n = 5; Fig. 6a). This response was antagonized by ICI 118551 and propranolol to yield log K_D values of −9.23 ± 0.05 and −9.34 ± 0.08 for ICI 118551 and propranolol, respectively. Salbutamol stimulated a response (logEC_{50} = −7.53 ± 0.04, 89.4 ± 2.3% isoprenaline maximum, n = 5; Fig. 6b). However, this salbutamol-induced response was antagonized by ICI 118551 and propranolol to yield K_D values of −9.66 ± 0.04 (n = 5) and −9.72 ± 0.04 (n = 4), respectively. Thus, under these conditions, the concentration of antagonists required to inhibit the isoprenaline-induced response was 2.2-fold greater for ICI 118551 ($p <$ 0.001, unpaired t test) and 2.4-fold greater for propranolol ($p < 0.01$) than that required to antagonize the salbutamol response.

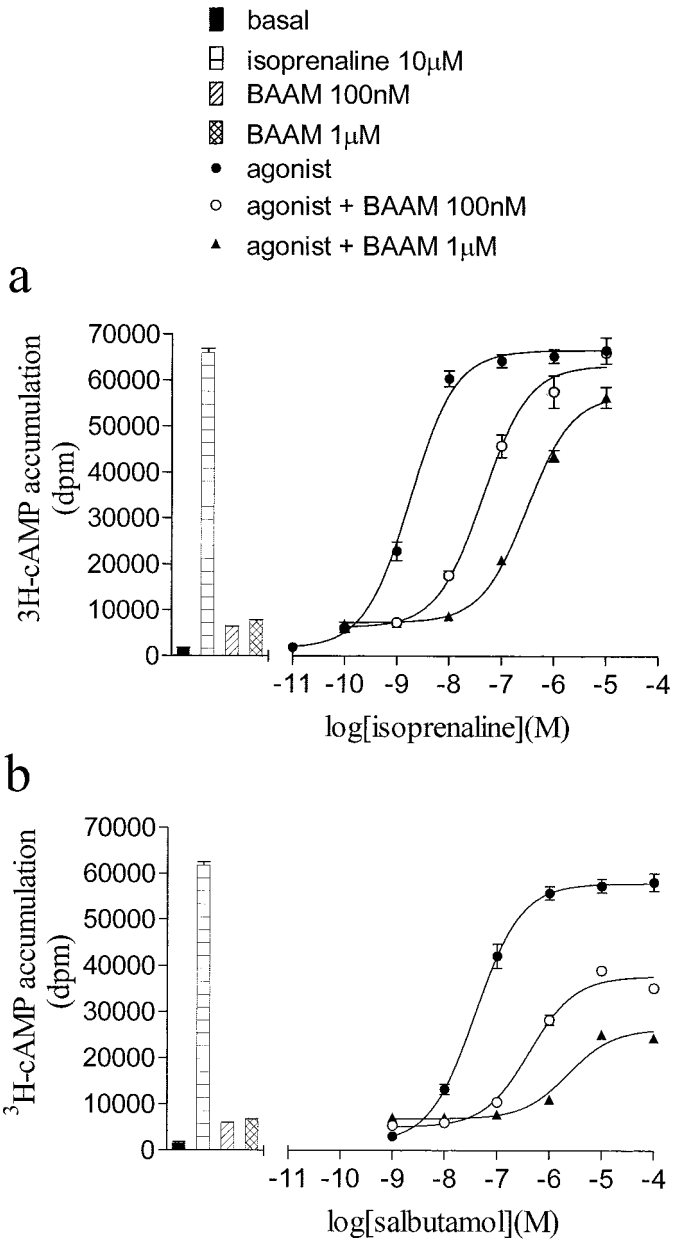

Fig. 4. [^{3}H]cAMP accumulation in response to isoprenaline (a) and salbutamol (b) after preincubation without and with 100 nM and 1 μM BAAM. Bars represent basal [^{3}H]cAMP accumulation, that in response to 10 μM isoprenaline alone, and that after preincubation with 100 nM and 1 μM BAAM alone. Data points are mean ± S.E.M. of triplicate determinations from a single experiments, and this is representative of four other experiments.

Similar K_D Values Obtained in CRE-Mediated Gene Transcription in CHO-mut β_2-SPAP Cells. CHO cells stably transfected with the CRE-SPAP reporter gene and a mutant β_2-adrenoceptor, in which the PKA and GRK (β-ARK) phosphorylation sites were mutated, were studied (CHO-$\beta_2$mut-SPAP). This receptor mutant has a reduced ability to be phosphorylated and internalized (Seibold et al., 2000). Isoprenaline (logEC_{50} −9.45 ± 0.14; Fig. 7a) induced a response in these cells 2.3 ± 0.13-fold higher than basal (n = 9). Salbutamol again appeared as a full agonist inducing a response 96.5 ± 3.3% of the isoprenaline maximum (log EC_{50} = −9.25 ± 0.08, n = 6) (Fig. 7b). These responses were antagonized by ICI 118551 to give log K_D values of −9.75 ± 0.09, n = 8, and −10.11 ± 0.12, n = 6, for isoprenaline and salbutamol, respectively. Thus, in these cells, in which phosphorylation is reduced and the receptor is slow to internalize, only 2.3-fold more antagonist was required to antagonize the isoprenaline response than the salbutamol response.

Lack of Responses in Native CHO-K1 Cells. Although there were increases in the [^{3}H]cAMP accumulation and CRE- SPAP production in response to forskolin, there were no responses to any of the above agonists in either assay, in CHO-K1 cells or cells transfected only with the CRE-SPAP reporter gene (CHO-SPAP cells) confirming the absence of any other β-adrenergic receptors in these cells (n = 4). These data confirm that the cAMP and SPAP responses to the β-agonists described above are dependent upon the presence of the transfected human β_2-adrenoceptor and not caused by the presence of any other receptor endogenously expressed in CHO cells.

Discussion

Antagonists, with little or no intrinsic efficacy of their own, should have an affinity for binding to a given receptor that remains constant irrespective of the agonist used or the functional readout measured (Kenakin et al., 1995). A change in antagonist affinity has previously been used as evidence for the presence of a different receptor (Arunlakshana and Schild, 1959; Black et al., 1965, 1972) or a second binding site (Kenakin and Boselli, 1989; Konkar et al., 2000). According to the ternary complex model, receptors exist in active (R*) and inactive (R) states. In the active state, receptors are coupled to G-proteins and have higher affinity for agonists

whereas in the inactive state, receptors are not coupled to G-proteins and have low agonist affinities (Leff, 1995). β_2-Adrenoceptors become phosphorylated, desensitized, and internalized after incubation with an efficacious β_2-agonist (Krupnick and Benovic, 1998). Also, the efficacy of the agonists dictates the extent to which phosphorylation and desensitization occur (Clark et al., 1999). Receptor phosphorylation uncouples the receptor-G-protein complex and thus reduces signaling (Krupnick and Benovic, 1998; Clark et al., 1999; Kohout and Lefkowitz, 2003). Thus, R* is phosphorylated by PKA to R^P. In addition, if a sufficiently highly efficacious agonist occupies enough receptors, the receptors can be further phosphorylated by GRKs to R^{PP}. Here, we have shown that the affinity of an antagonist for the human β_2-adrenoceptor changes in a time-dependent manner depending on the efficacy of the agonist used to measure it.

The major difference between the agonists used here is their efficacy. Isoprenaline and adrenaline are well known, very efficacious, full agonists, whereas salbutamol has been reported to be a partial agonist in many systems (e.g., BEAS-2B cells; January et al., 1998). Although in this CHO-β_2 cell system all four agonists appear as full agonists, the different efficacies were exposed when the irreversible β-adrenoceptor antagonist BAAM was used to effectively remove cell surface receptors in the cAMP assay. A similar pattern was seen with ICI 118551, suggesting that it is also a "sticky" ligand, removing β_2-adrenoceptors from the equilibrium. The less efficacious agonists, salbutamol, and terbutaline, need to occupy many receptors to induce a maximum response. Increasing ICI 118551 concentrations depletes the remaining pool of receptors, which soon become insufficient for a maximal response to be sustained and the maximum response achieved therefore decreased (Fig. 2 and 4). However, at these concentrations of ICI 118551, there were still sufficient receptors left for the efficacious ligands isoprenaline and adrenaline, which need to occupy very few receptors to induce a full response, to stimulate maximal responses. This effect was not seen in the CRE-gene transcription assays, in which a true equilibrium was probably reached over the 5-h incubation. A similar pattern was seen with another "sticky" ligand, CGP 12177, which has a dissociation half-life of 65 min (Baker et al., 2002a).

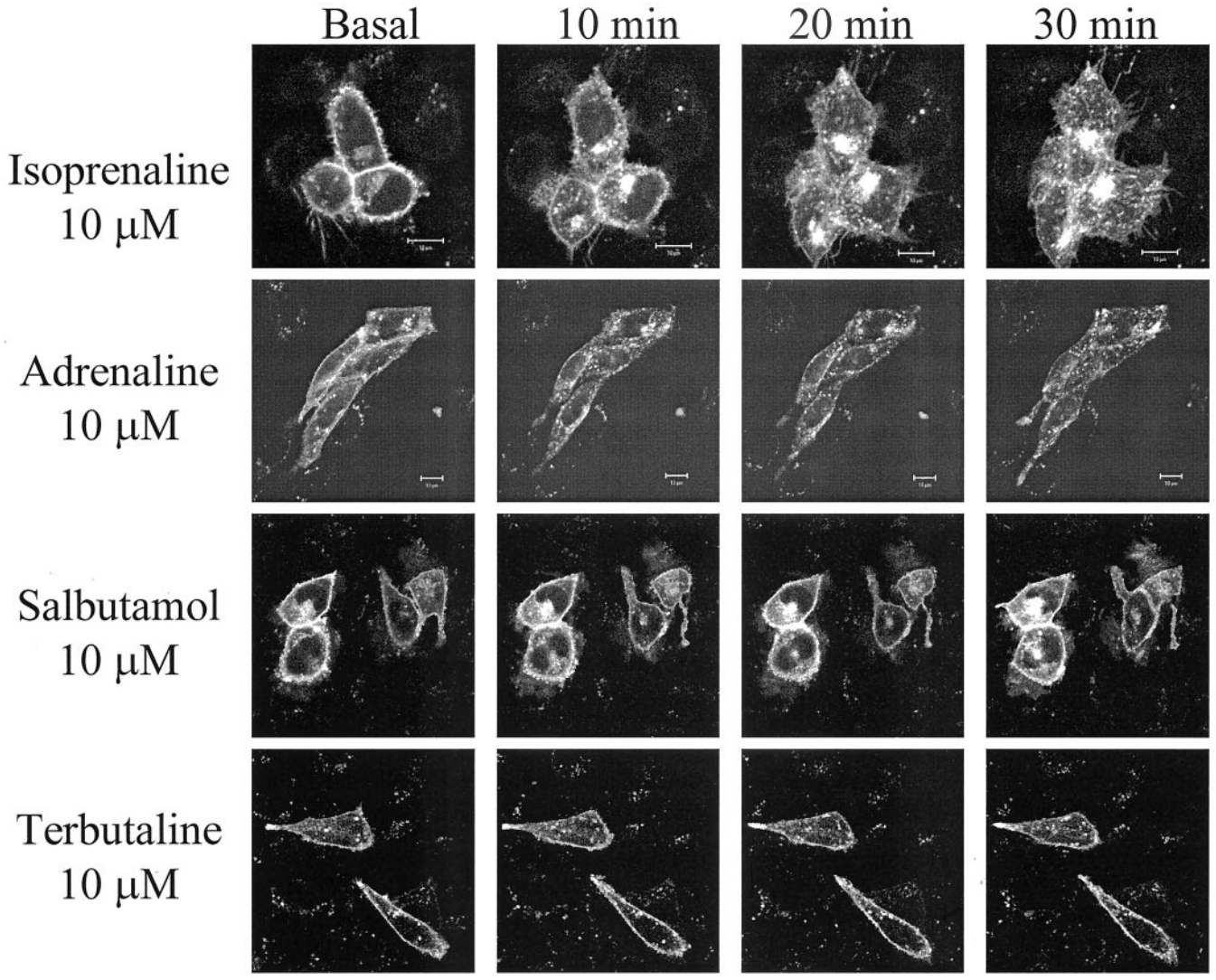

Fig. 5. Confocal images showing CHO-β_2-GFP cells under basal conditions and after 10-, 20-, and 30-min incubation (37°C) with 10 μM isoprenaline, 10 μM adrenaline, 10 μM salbutamol, and 10 μM terbutaline. Similar images were obtained when the concentrations of agonist were increased to 100 μM in all cases.

The major difference between these two assays is the length of agonist incubation time. In reporter gene assays, time must be allowed for transcription, translation, and protein assembly, and the agonist is usually present for the full incubation time (Rees et al., 1999). Indeed, an increase in gene transcription cannot be measured after only 10 min of agonist incubation (Baker et al., 2003). An indicator that the agonist efficacy and time of incubation are important is provided by the EC_{50} values of the agonist responses. The salbutamol and terbutaline responses are shifted leftward by 16.2- and 18.7-fold when moving from measuring the secondary messenger (cAMP) to the downstream gene transcription response (CRE-SPAP). Thus an increase in potency of these responses occurs, probably from amplification at the level of PKA. However, the concentration response curves for isoprenaline- and adrenaline-induced gene transcription are shifted 4.4- and 5.9-fold to the right; i.e., over time, the

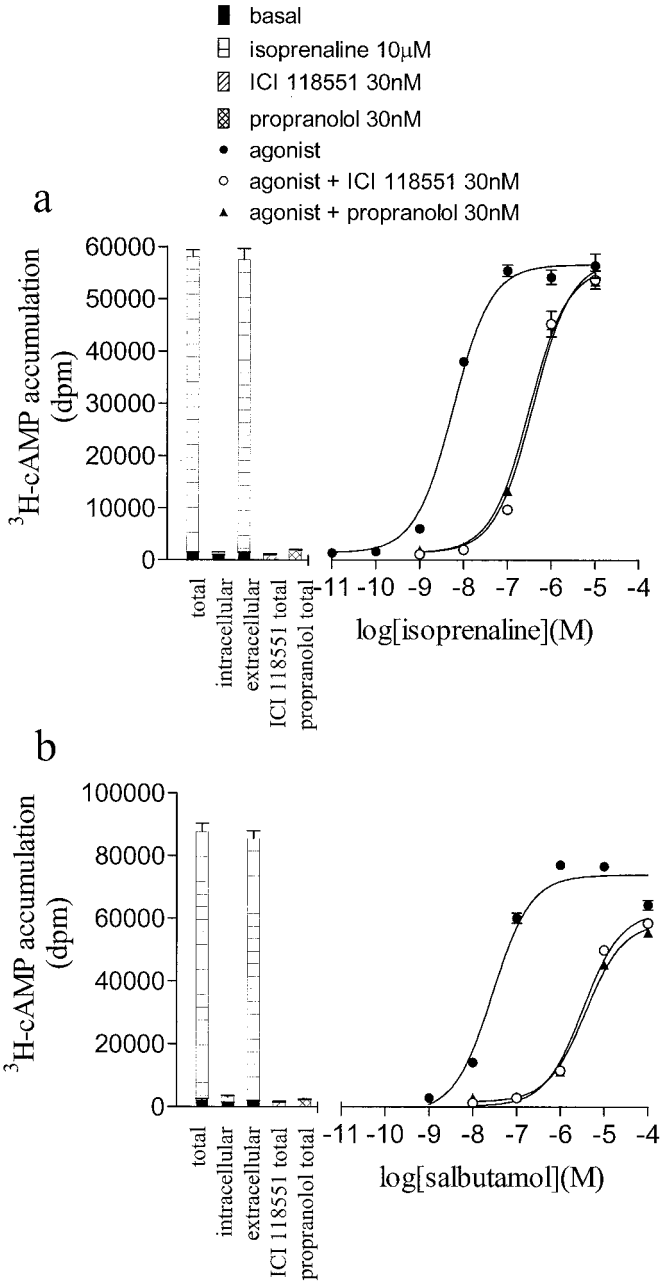

Fig. 6. Total [^{3}H]cAMP accumulation from CHO-β_2 cells after 5-h incubation with isoprenaline (a) and salbutamol (b) in the absence and presence of 30 nM ICI 118551 and 30 nM propranolol. Bars represent basal accumulation of total [^{3}H]cAMP over 5 h and that in response to 10 μM isoprenaline under the same conditions, that secreted into the extracellular media over the 5-h incubation (basal and in response to 10 μM isoprenaline), and that measured from inside the cells (intracellular [^{3}H]cAMP) at 5 h (basal and in response to 10 μM isoprenaline). Data points are mean ± S.E.M. of triplicate determinations, and these single experiments are representative of five (a) and four separate experiments (b).

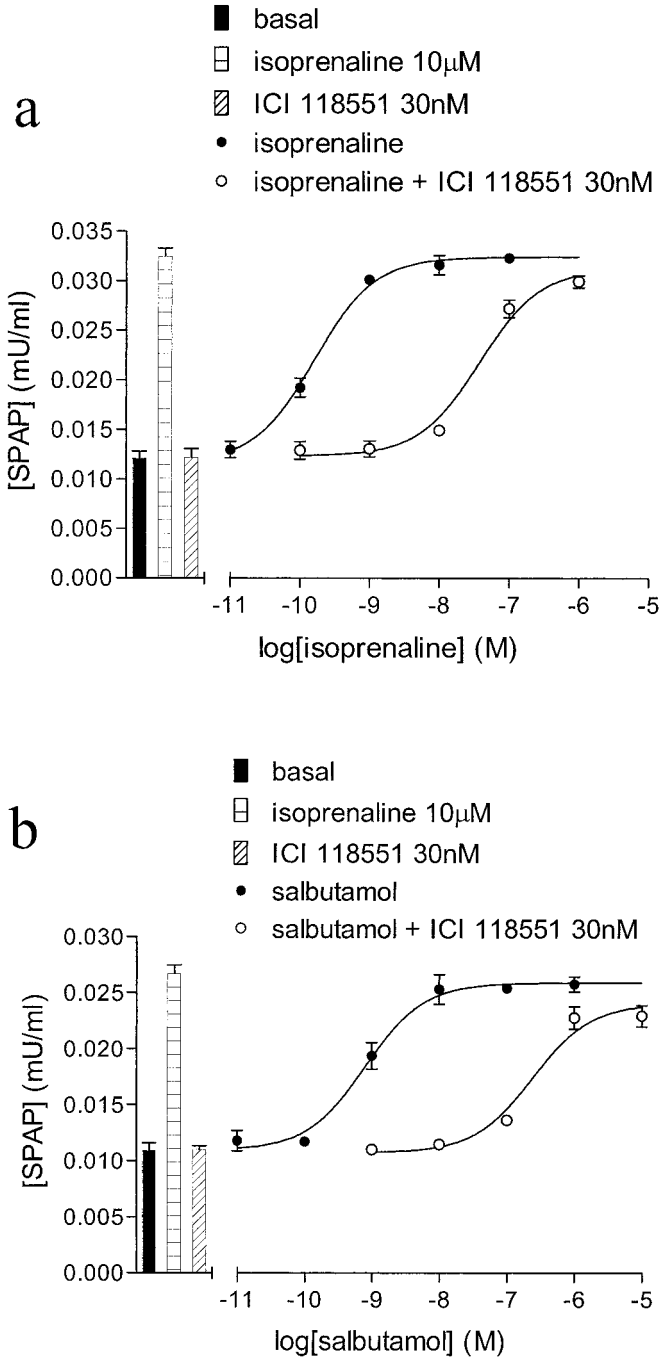

Fig. 7. SPAP production in response to a) isoprenaline and b) salbutamol in the presence and absence of 30 nM ICI 118551 in CHO-$\beta_2$mut-SPAP cells. Bars represent basal SPAP production and that in response to 10 μM isoprenaline and 30 nM ICI 118551 alone. Data points are mean ± S.E.M. of triplicate determinations and these single experiments are representative of eight (a) and six separate experiments (b).

responses became less potent. This isoprenaline-induced desensitization is also seen comparing the 10-min and 5-h cAMP accumulation responses: the 5-h isoprenaline EC_{50} value becomes right-shifted by 3.3-fold compared with the 10-min response, whereas the salbutamol EC_{50} value remains unchanged.

Although salbutamol- and adrenaline-induced PKA-mediated phosphorylation of the β_2-adrenoceptor is similar, the GRK (β-ARK) phosphorylation is much less for salbutamol than for the more efficacious agonist adrenaline (January et al., 1998). It may be that this GRK-mediated phosphorylation and internalization caused the decrease in potency with isoprenaline and adrenaline over time. Within the first few minutes of isoprenaline or adrenaline addition, the β_2-adrenoceptors will become phosphorylated by both GRKs and PKA, and a new steady state of receptor internalization and recycling to the membrane would ensue, with many of the membrane receptors in the phosphorylated state (R^{PP}). This would lead to the rightward shift of the concentration-response curve as a consequence of G-protein uncoupling and receptor internalization. The different efficacies of the four agonists were also apparent in the internalization experiments with the GFP-tagged human β_2-adrenoceptor. Here, isoprenaline and adrenaline clearly internalized the receptor within 30 min, whereas salbutamol and terbutaline did not.

In the cAMP accumulation experiments (10-min agonist incubations), the antagonist affinities (ICI 118551, propranolol, or atenolol) are the same regardless of the agonist used. This is as expected for antagonism at a single receptor (Kenakin et al., 1995). However, in the reporter gene assay, the K_D value for each of the three antagonists was 10-fold greater when isoprenaline and adrenaline were the agonists than when salbutamol and terbutaline were present. This suggests that either the antagonist or the receptor has become altered in some way. It is unlikely that an antagonist chemical reaction is the cause, because the same drugs were used in all experiments in this study. It seems, therefore, that the chemical nature of the receptor changes over time; i.e., isoprenaline and adrenaline induce a change in the β_2-adrenoceptor (that salbutamol and terbutaline do not) such that the binding affinity of the antagonists is reduced. It is therefore possible that the R^{PP} GRK phosphorylated receptors have a lower affinity for β-adrenoceptor antagonists.

Further confirmation that the change in antagonist affinity is time-related comes from the 5-h cAMP accumulation experiments. Because it was not possible to measure intracellular [^{3}H]cAMP at 5 h (Fig. 6) (possibly because the intracellular [^{3}H]substrate had been depleted by this time or because of induction of endogenous phosphodiesterases), total cAMP accumulation over the full 5 h was measured (i.e., that secreted into the media over the whole 5 h plus any intracellular cAMP). Although this is a total 5-h accumulative measure, and therefore unlike the rate of gene transcription at 5 to 6 h, the antagonist K_D values for isoprenaline- and salbutamol-stimulated responses are significantly different and show a move toward that observed with measurements of gene transcription. Thus, the change in antagonist affinity over time is again seen.

The role of receptor phosphorylation was examined using CHO cells stably expressing a mutant β_2-adrenoceptor in which the GRK and PKA phosphorylation sites have been mutated, such that receptor phosphorylation and internalization is reduced (Seibold et al., 2000). At the wild-type β_2-adrenoceptor, isoprenaline was more potent than salbutamol in the short-term cAMP assay but less potent in the long-term cAMP and gene transcription assays because of isoprenaline-induced receptor phosphorylation and internalization. In the mutant receptor gene transcription assay, however, isoprenaline was again more potent than salbutamol. Thus, there seems to be a lack of isoprenaline-induced desensitization with the mutant receptor. Furthermore, the differences in antagonist affinities seen with this mutant receptor were also reduced: the affinity of ICI 118551 in the presence of isoprenaline was only 2.3 times greater than that obtained when salbutamol was present (rather than the order of magnitude seen with the native receptor). Thus the difference in antagonist affinity in these receptors with reduced phosphorylation is less than that seen with wild-type receptors, suggesting that isoprenaline-induced receptor phosphorylation is indeed involved in changing antagonist affinity.

This observation raises important questions about using reporter gene assays in high-throughput screening for drug discovery. Efficacious agonists (e.g., isoprenaline) are often used as the standard from which antagonist affinities are measured. This study suggests that in long-term assays, less efficacious or partial agonists that do not alter the affinity state of the receptor would provide better standards (i.e., agonists incapable of inducing the R^{PP} state). Certainly, comparisons of antagonist affinity made using agonists of different efficacies in long-term assays should be interpreted with care.

In summary, we have shown that the relative position of the agonist concentration responses change depending on the efficacy of the agonist and the time of agonist incubation. Furthermore, antagonist affinity also depends on both the agonist used and the length of time of agonist incubation. With longer incubation times (e.g., as required for reporter gene assays), very efficacious agonists induce changes in the β_2-adrenoceptor that reduce antagonist affinity that less efficacious agonists do not. Therefore, high agonist efficacy seems to induce a chemical modification of the receptor that reduces the antagonist-receptor affinity. For future high throughput screening in drug discovery, less efficacious or even partial agonists may therefore be more reliable than the traditional full efficacious agonists when using reporter gene techniques to estimate antagonist affinity.

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