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Temporal Characteristics of cAMP Response Element-Mediated Gene Transcription: Requirement for Sustained cAMP Production

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ABSTRACT

Many clinically used drugs are G-protein–coupled receptor (GPCR) antagonists and are given long-term to prevent receptor activation by endogenous agonists. Most GPCR antagonists are considered to have little agonist efficacy of their own. However, many β antagonists do stimulate very small β_2 adrenoceptor-mediated cAMP responses, but these responses become substantial at the level of cAMP response element (CRE)-gene transcription. Here, we compared the temporal characteristics of these β_2 adrenoceptor-mediated cAMP and CRE-gene transcription responses with ligands of differing agonist efficacy. Within a minute, full agonists (e.g., isoprenaline) stimulated large increases in intracellular and exported cAMP. Very weak partial agonists (e.g., alprenolol) did not increase intracellular cAMP (only stimulating a small export). However, all agonists (regardless of efficacy) stimulated an increase in CRE-gene transcription after a 2-h incubation. An initial 30-min continual stimulation was required to initiate the process of CRE-gene transcription for all ligands. Longer agonist incubations resulted in larger gene transcription responses in a proportional manner for both weak and full agonists alike, and this was despite the lack of intracellular cAMP detection for the weaker ligands. Thus, the major initiator for CRE-gene transcription was not cAMP concentration or total quantity generated but a sustained turnover of intracellular cAMP and hence sustained stimulation of CREB phosphorylation. Thus, long-acting agonists and long-term treatments with very weak partial agonists (including many drugs classified previously as antagonists based on traditional second-messenger assays, e.g., several clinically used "β-blockers") may cause more substantial gene transcription than previously believed.

Many drugs used in clinical practice are antagonists of G-protein–coupled receptors (GPCRs), including β-blockers for the treatment of hypertension, ischemic heart disease, and heart failure (Heidenreich et al., 1999; Wright, 2000, Morgan et al., 2001; Tendera and Ochala, 2001); muscarinic antagonists for asthma and chronic obstructive airways disease (Barnes et al., 1995; Chapman, 1996); histamine H_1 antagonists for allergy (Van Cauwenberge, 2002; Winbery and Lieberman, 2002); histamine H_2 antagonists for the reduction of gastric acid secretion (Pattichis and Louca, 1995); and α antagonists for hypertension and benign prostatic hyperplasia (Fulton et al., 1995; Pool, 1996). Many of these drugs are considered to have no significant efficacy of their own and are given to prevent the binding of endogenous agonists (e.g., adrenaline and histamine) to their receptors and thus prevent receptor activation. However, several studies have now shown that many clinically used "antagonists" are not neutral but actually have low efficacy, either positive (giving rise to partial agonist effects) or negative (inverse agonists), at their respective receptors (Chidiac et al., 1994; Smit et al., 1996; Baker et al., 2003a,b).

Traditionally, the pharmacology of many GPCR agonists and antagonists has been determined by following their effects on short-term responses, such as using changes in second-messenger levels or contractile activity (Chidiac et al., 1994; Adamson et al., 2000; Saitoh et al., 2002). Recently, however, we have shown that the magnitude of the response measured depends greatly on which downstream response is being examined. For example, agonist responses to β-blockers (even in the presence of a phosphodiesterase inhibitor) are small at the level of cAMP accumulation but very substantial (in the absence of a phosphodiesterase inhibitor) at the level of gene transcription (Baker et al., 2003a,b).

In clinical practice, most antagonists are given to achieve

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ABBREVIATIONS: GPCR, G-protein–coupled receptor; CRE, cAMP response element; CREB, cAMP response element binding protein; FRA-1, fos-related antigen 1; PKA, protein kinase A; SPAP secreted placental alkaline phosphate; CHO, Chinese hamster ovary; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; ICI 118551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol.

long-term blockade of the receptor in question. For example, β-blockers are given to reduce the stroke risks of hypertension and the painful symptoms of angina, but to achieve 24-h reduction in symptoms or risk, 24-h receptor blockade is required (e.g., using multiple dosing regimes, slow-release preparations, or slowly dissociating drugs). Thus, the short-term context in which the pharmacology of the antagonists was originally established is not the same as the clinical context in which they are used. The very low efficacy of many clinically used antagonists may therefore cause more substantial clinical effects than previously believed, such as at the level of gene transcription.

Reporter gene assays, which are based on gene transcription, are increasingly being used, in both academia and industry, to study the pharmacology of agonists and antagonists of GPCRs (Hill et al., 2001). Here, cell lines are constructed containing the receptor of interest and a sequence of DNA encompassing a promoter element upstream of a sequence encoding a unique protein. Thus, stimulation of the surface receptor (e.g., β_2 adrenoceptor) causes a change in the concentration of an intracellular second messenger (e.g., an increase in intracellular cAMP). This in turn (e.g., via activation of PKA and CREB) activates the response element of the promoter (e.g., CRE) to initiate gene transcription (Rees et al., 1999; Hill et al., 2001). However, the nature of these gene transcription assays means that several hours are required for the transcription and translation of the gene and assembly of an active protein product in sufficient quantities to be measurable (Rees et al., 1999). Typically, 4 to 6 h are allowed for this process, and for ease, both the agonists and antagonists are invariably left in contact with the cells for the whole of the incubation time (Rees et al., 1999). However, the actual temporal characteristics of this process have not been established. There is plenty of evidence that intracellular cAMP increases rapidly (within minutes of agonist addition) in cells, but it remains to be established whether the initiation of gene transcription responds to the concentration or total quantity of the cAMP response generated or whether a prolonged stimulus is required.

This study describes the temporal characteristics of cAMP production and CRE-mediated gene transcription in response to full agonists and low-efficacy β-blockers. We provide evidence that the driving mechanism for CRE-gene transcription is a time-dependent sustained production of cAMP, and hence sustained phospho-CREB stimulation, and that the actual concentration of intracellular cAMP required is exceedingly low.

Materials and Methods

Materials. Cell culture reagents were from Sigma Chemical (Poole, Dorset, UK) except for fetal calf serum, which was from PAA Laboratories (Teddington, Middlesex, UK). [^{3}H]adenine, [^{14}C]cAMP, and enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Salmeterol was a gift from Dr. Malcolm Johnson (GlaxoSmithKline, Uxbridge, Middlesex, UK). CGP 12177 was from Tocris Cookson Inc. (Avonmounth, Bristol, UK). The Luclite Plus Assay System was from PerkinElmer Life and Analytical Sciences (Groningen, the Netherlands). Primary antibodies to c-fos–K25 [a pan c-fos/fos-related antigen 1 (FRA-1) antibody], the selective c-fos-4 and selective FRA-1 antibodies were obtained from Autogen-Bioclear UK (Calne, Wiltshire, UK). The primary antibodies phospho-CREB and total CREB and the secondary antibody (peroxidase-conjugated donkey anti-rabbit immunoglobulins) were from New England Biolabs (Hitchin, Hertfordshire, UK). Sigma Chemical supplied all other reagents.

Cell Culture. Experiments [CRE-gene transcription (secreted placental alkaline phosphatase, SPAP), cAMP, and Western blots] were performed using a stable cells line of CHO cells expressing both the human β_2 adrenoceptor and a SPAP reporter gene under the transcriptional control of a six-CRE promoter (CHO-β2-SPAP cells; 300–400 fmol/mg protein) (McDonnell et al., 1998). The same clonal β_2 adrenoceptor-expressing cell line (without the SPAP reporter) was also secondarily transfected with a luciferase reporter gene regulated by the human c-fos promoter, essentially as described by Megson et al. (2001), and used for c-fos–gene transcription (luciferase) experiments (CHO-β2-c-fos–luciferase cells). All CHO cells were grown at 37°C in Dulbecco's modified Eagle's medium/Ham's F12 nutrient mix containing 10% fetal calf serum and 2 mM L-glutamine in a humidified 5% CO_2/95% air atmosphere.

[^{3}H]cAMP Accumulation. All experiments were performed in Dulbecco's modified Eagle's medium/Ham's nutrient mix F12 containing 2 mM L-glutamine (serum-free media). Cells were grown to confluence in 24-well plates then prelabeled with 1 ml of [^{3}H]adenine

TABLE 1

Log EC_{50} values for total and extracellular ^{3}H-cAMP measurements after incubation with a range of agonists for 10 min to 5 h

Values are means ± S.E.M. of n separate experiments.

	Log EC_{50}	% Isoprenaline	n
Total [^{3}H]cAMP after 60 min in the presence of 1 mM IBMX[a]			
Isoprenaline	−7.97 ± 0.07	100	4
Salmeterol	−9.52 ± 0.04	90.9 ± 2.0	10
Alprenolol	−9.09 ± 0.14	3.23 ± 0.17	4
CGP 12177	−9.05 ± 0.10	3.19 ± 0.08	4
Total [^{3}H]cAMP after 60 min in the absence of IBMX[b]			
Isoprenaline	−8.15 ± 0.08	100	4
Alprenolol	−9.45 ± 0.13	2.9 ± 0.29	4
Total [^{3}H]cAMP after 10 min in the absence of IBMX[c]			
Isoprenaline	−7.84 ± 0.09	100	4
Alprenolol	−9.71 ± 0.29	5.6 ± 1.2	4
Extracellular (secreted) [^{3}H]cAMP after 5 h in the absence of IBMX[d]			
Isoprenaline	−7.80 ± 0.05	100	4
Salmeterol	−9.65 ± 0.03	94.3 ± 3.0	4
Alprenolol	−9.26 ± 0.20	5.64 ± 0.56	4
CGP 12177	−9.62 ± 0.10	4.60 ± 0.6	7

[a] Isoprenaline response was 38.1 ± 3.6-fold over basal (60 min, plus IBMX). Mean basal dpm was 7426.
[b] Isoprenaline response was 42.7 ± 4.6-fold over basal (60 min, no IBMX). Mean basal dpm was 1758.
[c] Isoprenaline response was 33.6 ± 2.3-fold over basal (10 min, no IBMX). Mean basal dpm was 1407.
[d] Isoprenaline response was 14.4 ± 1.3-fold over basal (5 h total secreted ^{3}H-cAMP). Mean basal dpm was 1999.

(2–4 μCi/ml) for 2 h at 37°C. The [^{3}H]adenine was removed, and each well was washed by the addition and removal of 1 ml of serum-free media. A fresh 3-ml sample of serum-free media was then added to each well. Where used, IBMX (1 mM) was added and incubated for 30 min before the addition of agonist. Agonists in 10 μl were then added, and the cells were incubated for 1 min to 5 h. Where total [^{3}H]cAMP was measured, the reaction was terminated by the addition of 50 μl of concentrated HCl. [^{3}H]cAMP was separated from other [^{3}H]adenine nucleotides by sequential Dowex and alumina chromatography, and each column was corrected for efficiency by comparison with [^{14}C]cAMP recovery as described previously (Baker et al., 2002). Intracellular and extracellular [^{3}H]cAMP were measured separately where stated. Here, after the given agonist incubation time, the extracellular media were removed into a separate 24-well plate for measurement of extracellular-secreted cAMP. The cells were washed twice by the addition and removal of 2 × 1 ml of serum-free media. A third 1-ml sample of serum-free media was then added to the well (intracellular cAMP measured). Concentrated HCl (50 μl) was then added to all wells (both the washed "intracellular [^{3}H]cAMP" wells and the extracellular-secreted [^{3}H]cAMP wells). Column separation of [^{3}H]cAMP from other [^{3}H]adenine nucleotides was then performed as described previously (Baker et al., 2002).

CRE-Mediated Gene Transcription: SPAP. Cells were grown to confluence in 24-well plates and then in serum starved for 24 h before experimentation in serum-free media. On the day of experimentation, the media were removed and replaced with 1 ml of fresh serum-free media. Agonists (in 10 μl, each condition in triplicate) were added and incubated for various periods between 10 min and 5 h at 37°C in a humidified atmosphere of 5% CO_2/95% air.

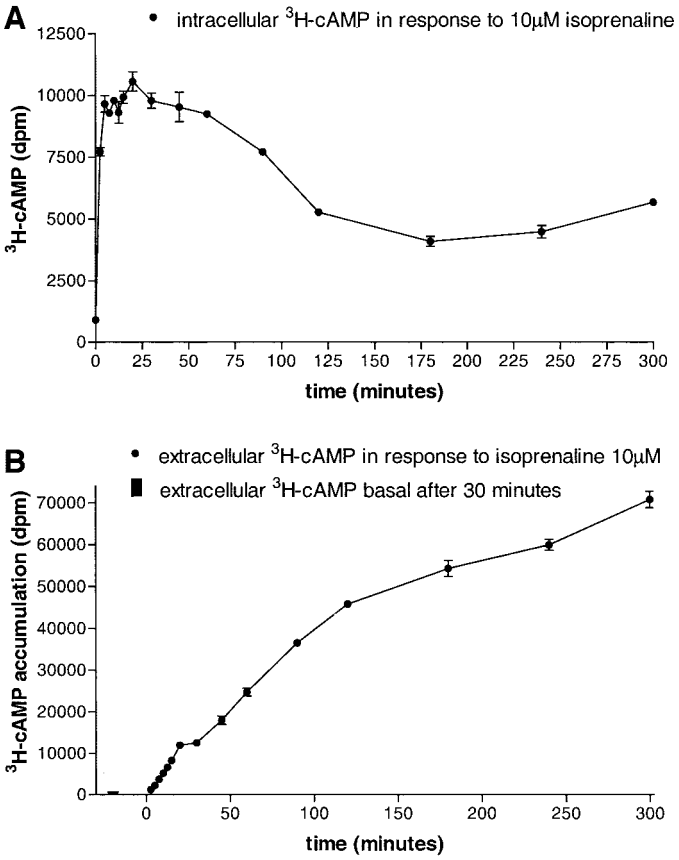

Fig. 1. Levels of intracellular (a) and extracellular (b) [^{3}H]cAMP in response to 10 μM isoprenaline measured over 0 to 5 h. These experiments were performed in serum-free media without IBMX. b, bar represents basal [^{3}H]cAMP after 30 min. Data points are mean ± S.E.M. from triplicate determinations from single experiments and these are representative of eight separate experiments in each case.

Where intracellular SPAP was measured after the set time of agonist incubation (10 min to 5 h), the media and agonists were removed from the wells, and 300 μl of distilled water was added to lyse the cells. The plates were then frozen at −80°C for at least 30 min before thawing and then centrifuged at 1000 rpm for 5 min. A 20-μl sample of the supernatant was sampled and the SPAP activity was determined (see below). Where total secreted SPAP was measured, 20 μl of media was sampled from each well at set times (a separate plate for each time point) from the addition of the agonist (total secreted SPAP over time since agonist addition) and assayed for SPAP content. Finally, where the rate of gene transcription was measured, after a total of 5 h of incubation, the serum-free media and any remaining drugs were removed, and 300 μl of serum-free media was added and incubated for 1 h (37°C). This was sampled (20 μl) and SPAP activity was determined, thus measuring SPAP secretion over hour 5 to 6 from the addition of agonist.

The 20-μl samples obtained from the various measures above were transferred to 96-well plates and heated to 65°C for 30 min to destroy any endogenous alkaline phosphatases. SPAP reporter activity was then quantified by following the color change caused by the hydrolysis of *p*-nitrophenol phosphate. *p*-Nitrophenol phosphate (5 mM) in diethanolamine buffer (200 μl) was added to each sample and incubated at 37°C in air for 1 h. The plates were then read at 405 nm using an MRX plate reader (Dynatech Labs, Chantilly, VA), and the data were converted to SPAP concentration mU/ml as described previously (McDonnell et al., 1998).

c-fos–Mediated Gene Transcription: Luciferase. Cells were grown to confluence in white 96-well plates and then incubated in

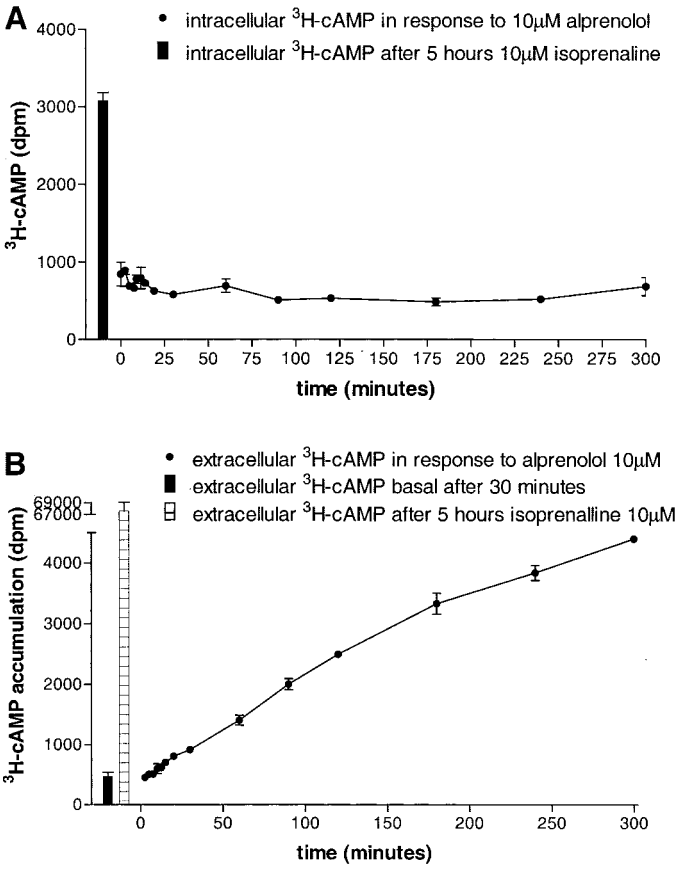

Fig. 2. Levels of intracellular (a) and extracellular (b) [^{3}H]cAMP in response to 10 μM alprenolol measured over 0 to 5 h. These experiments were performed in serum-free media without IBMX. Bars represent basal [^{3}H]cAMP at 30 min and that in response to 10 μM isoprenaline at 5 h. Data points are mean ± S.E.M. of triplicate determinations from single experiments and these are representative of four separate experiments in each case.

serum-free media (200 μl/well) for 24 h before experimentation. On the day of experimentation, agonist (in 20 μl or serum-free media to the control wells) was added to the wells, and the cells were incubated for 5 h at 37°C. The drugs and media were then removed, and the cells were washed twice with 200 μl of phosphate-buffered saline (PBS). PBS (2 ml) containing 1 mM Ca^{2+}/1 mM Mg^{2+} was mixed with 2 ml Luclite Plus; 40 μl of this was added to each well, and the plate was counted on a Topcount (PerkinElmer Life and Analytical Sciences).

Measurement of Phospho-CREB, Total CREB, c-fos, and FRA-1 Using Western Blotting. Cells were grown to confluence in 6-well plates, washed, then incubated in serum-free media for 24 h. Agonist (or serum-free media to the control wells) was then added to the wells, and the cells were stimulated for 0 to 5 h at 37°C. After the set agonist incubation time, the cells were washed with 1 ml of ice-cold PBS and then 200 μl of ice-cold lysis buffer [20 mM Tris/HCl, pH 7.4, 1 mM EGTA, 0.5% (v/v) Triton X-100, 1 mM NaF, 1 mM dithiothreitol, 70 mM β-glycerophosphate, and 10 μl protease inhibitor cocktail (Sigma)/5 ml buffer] added to each well. Cells were then scraped from the wells, and the solubilized protein content was analyzed by the method used by Lowry et al. (1951). Laemmli sample buffer (Sigma) was added to each sample [1:1 (v/v)], boiled at 95°C for 5 min and then subjected to Western blot analysis. Protein samples (50 μg/well) were separated by SDS-polyacrylamide gel electrophoresis (11.25% acrylamide gel) using the mini protein-II system (Bio-Rad, Hemel Hempstead, UK). After transfer of the proteins to a nitrocellulose membrane, the membranes were blocked for 1 h in 5% (w/v) low-fat dried milk in TBST (25 mM Tris, 125 mM NaCl, and 0.1% Tween 20, pH. 7.6) at room temperature. Blots were then incubated with primary antibody [anti-phospho-CREB (Ser133), anti-CREB, anti–c-fos–K25 (for c-fos and FRA-1), anti–c-fos-4 (for c-fos only), or anti–FRA-1] for 2 h at room temperature in TBST containing 3% bovine serum albumin. The blots were washed and incubated with the secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody for 1 h at room temperature in 3% bovine serum albumin/TBST. The secondary antibody was then removed, the membranes were washed, and the blots were developed using the enhanced chemiluminescence detection system (Amersham).

Data Analysis. A 10 μM (maximal) isoprenaline concentration was included (in triplicate) in each plate for each separate experiment for [^{3}H]cAMP accumulation, CRE-SPAP gene transcription, and c-fos–luciferase gene transcription to allow agonist responses to be expressed as a percentage of the isoprenaline maximum for each experiment. Sigmoidal agonist concentration-response curves were fitted to the following equation through computer-assisted nonlinear regression using the program Prism 2 (GraphPad Software Inc., San Diego, CA):

$$\text{Response} = \frac{E_{max} \times 10^{\log[A]}}{10^{\log EC_{50}} + 10^{\log[A]}}$$

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. Concentration-response curves for isoprena-

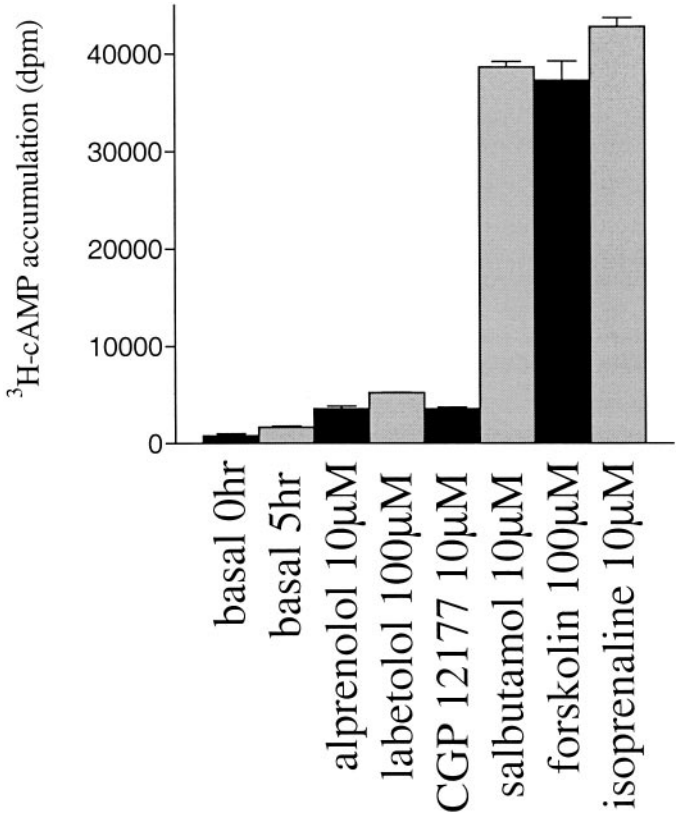

Fig. 3. Extracellular [^{3}H]cAMP measured 5 h after the addition of ligands. These experiments were performed in serum-free media without IBMX. Bars represent mean ± S.E.M. of triplicate determinations from single experiments which are representative of four separate experiments.

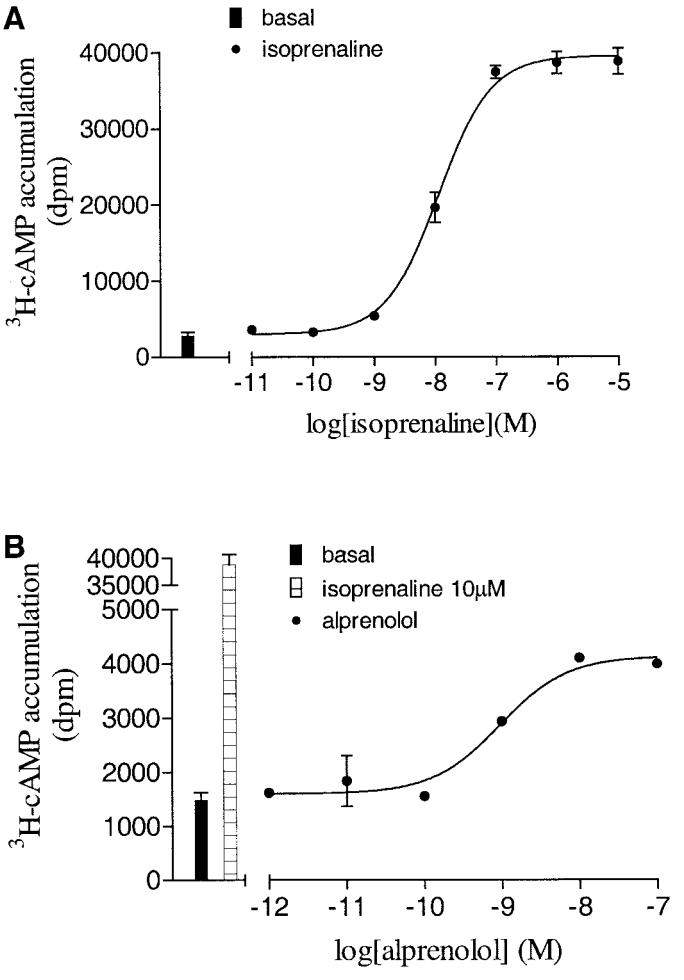

Fig. 4. Extracellular [^{3}H]cAMP measured 5 h after the addition of agonist in response to isoprenaline (a) and alprenolol (b). These experiments were performed in serum-free media without IBMX. Bars represent extracellular basal [^{3}H]cAMP accumulation and that in response to 10 μM isoprenaline measured after 5 h. Data points are mean ± S.E.M. of triplicate determinations from single experiments, and these are representative of four separate experiments in each case.

line and salbutamol (Figs. 6 and 7) were also fitted to two sites with Prism 2 using the equation

$$\%\ \text{maximal stimulation} = \frac{[A]N}{([A] + EC_{50}1)} + \frac{[A](100 - N)}{([A] + EC_{50}2)}$$

where N is the percentage of site 1, $[A]$ is the concentration of agonist, and $EC_{50}1$ and $EC_{50}2$ are the respective EC_{50} values for the two agonist sites. All data are presented as mean ± S.E.M. of triplicate determinations, and n in the text refers to the number of separate experiments.

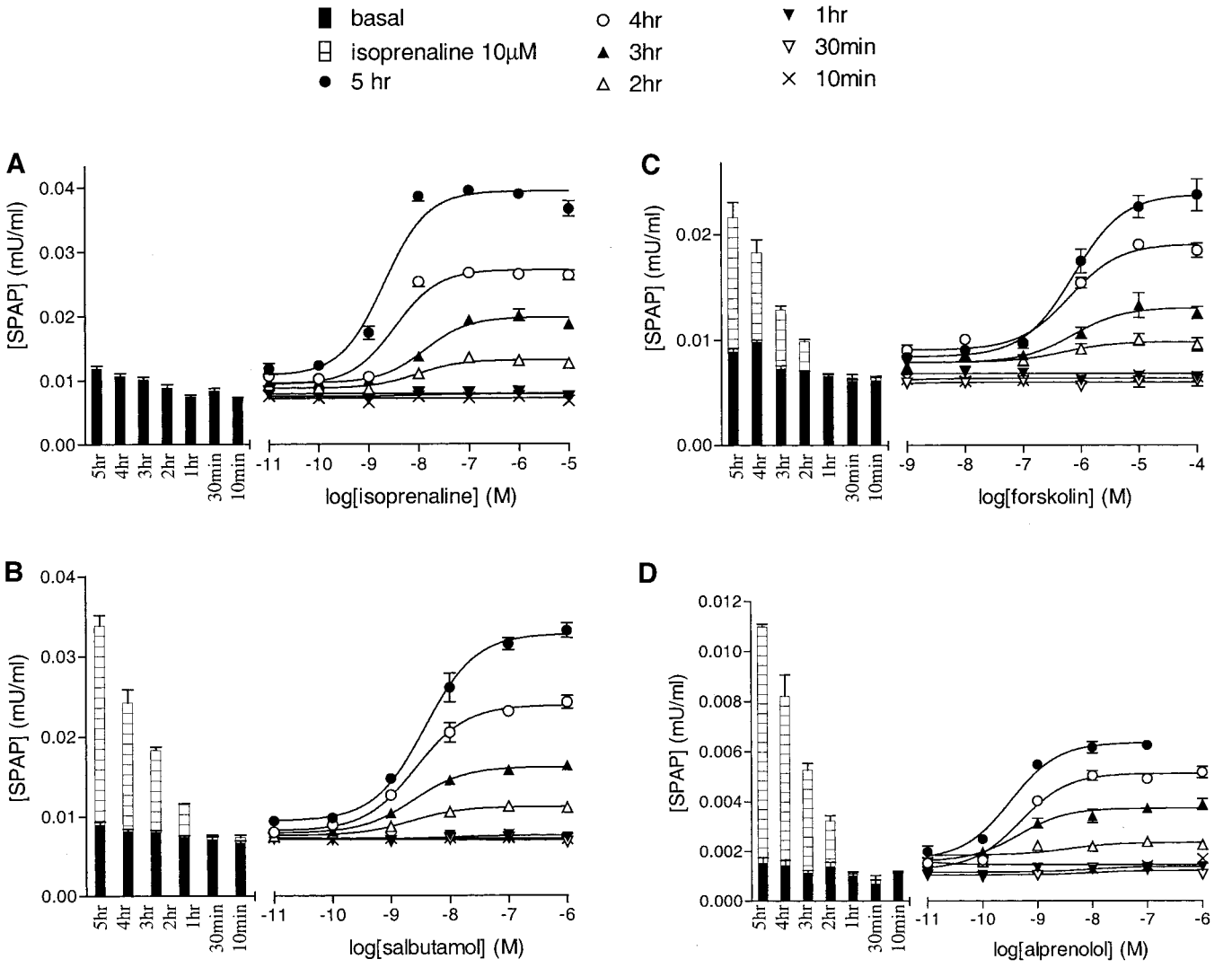

Fig. 5. Intracellular CRE-SPAP production in response to isoprenaline (a), salbutamol (b), forskolin (c), and alprenolol (d). Cells were lysed, and intracellular SPAP was measured 10 min to 5 h after the addition of agonist. Bars represent basal intracellular SPAP and intracellular SPAP after incubation with 10 μM isoprenaline at each time point. Data points are mean ± S.E.M. of triplicate determinations and these single experiments are representative of eight (a), three (b), three (c), and three (d) separate experiments.

TABLE 2

Percentage maximum responses for the production of intracellular SPAP after incubation with a range of agonists

Agonists were incubated for 10 min to 5 h before the cells were lysed and intracellular SPAP was measured. Values represent means ± S.E.M. of n determinations.

Agonist	n	% Isoprenaline	% of Maximal Agonist Response			
		5 h	4 h	3 h	2 h	1 h
Isoprenaline	8	100	68.5 ± 3.3	45.2 ± 3.3	21.4 ± 1.8	N.R.
Forskolin	3	116.7 ± 4.7	60.6 ± 2.7	33.2 ± 2.4	13.4 ± 0.8	N.R.
Salmeterol	4	98.4 ± 9.50	75.5 ± 3.5	52.5 ± 4.6	30.2 ± 7.6	N.R.
Salbutamol	3	94.7 ± 2.9	67.4 ± 2.4	35.3 ± 0.6	17.1 ± 3.1	N.R.
Labetolol	3	75.7 ± 1.0	66.3 ± 5.2	41.6 ± 2.2	21.4 ± 3.5	N.R.
Alprenolol	3	50.9 ± 4.3	77.2 ± 6.5	42.3 ± 4.8	13.5 ± 5.7	N.R.
CGP 12177	4	42.9 ± 4.2	65.2 ± 2.4	38.4 ± 4.7	17.4 ± 3.2	N.R.

% isoprenaline, maximum 5-h response of each ligand expressed as a percentage of the maximum 5-h response to 10 μM isoprenaline as determined in each experiment; % of maximal agonist response, response of that ligand expressed as a percentage of the maximum 5-h response for that same ligand; N.R., no responses were seen after 10-, 30-, and 60-min agonist incubation (see Fig. 5).

TABLE 3

Log EC_{50} values for intracellular SPAP production after incubation with a range of agonists.

Agonists were incubated for 10 min to 5 h before the cells were lysed and intracellular SPAP was measured. Values are means ± S.E.M. of n separate experiments.

Agonist	n	Log EC_{50} Values				
		5 h	4 h	3 h	2 h	1 h
Isoprenaline	8	−8.70 ± 0.21	−8.19 ± 0.25	−7.94 ± 0.23	−7.79 ± 0.24	N.R.
Forskolin	3	−6.02 ± 0.08	−6.08 ± 0.09	−6.27 ± 0.15	−6.47 ± 0.11	N.R.
Salmeterol	4	−10.41 ± 0.19	−10.25 ± 0.10	−10.25 ± 0.34	−10.04 ± 0.27	N.R.
Salbutamol	3	−8.54 ± 0.09	−8.57 ± 0.15	−8.72 ± 0.06	−8.35 ± 0.20	N.R.
Labetolol	3	−8.75 ± 0.08	−8.67 ± 0.14	−8.70 ± 0.02	−8.93 ± 0.22	N.R.
Alprenolol	3	−9.38 ± 0.06	−9.30 ± 0.11	−9.24 ± 0.15	−8.51 ± 0.45	N.R.
CGP 12177	4	−9.62 ± 0.23	−9.39 ± 0.28	−9.36 ± 0.07	−9.59 ± 0.16	N.R.

N.R., no responses were seen after 10-, 30-, and 60-min agonist incubation.

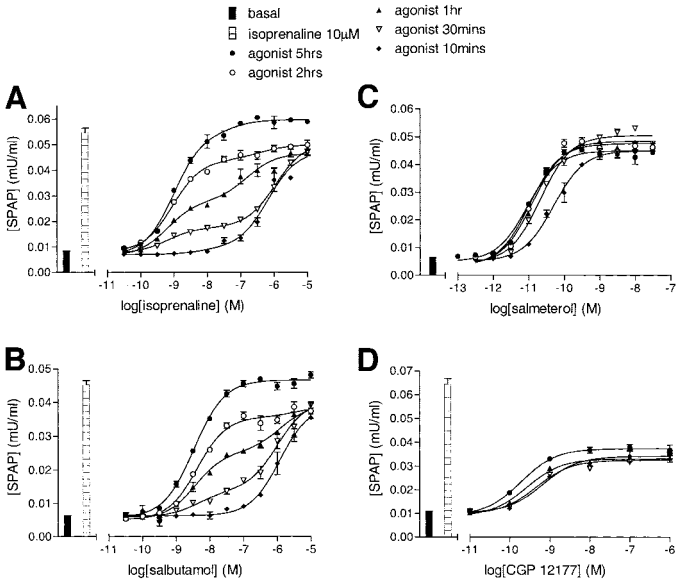

Fig. 6. Rate of CRE-SPAP secretion at 5 to 6 h after the addition of agonist in response to isoprenaline (a), salbutamol (b), salmeterol (c), and CGP 12177 (d). The agonist was added for 10 min to 5 h and then washed away, and the cells were incubated for the remainder of the 5 h in the absence of agonist. The rate of SPAP secretion between 5 and 6 h was then measured. Bars represent the basal rate of SPAP secretion and that after a 5-h incubation with 10 μM isoprenaline. Data points are mean ± S.E.M. of triplicate determinations and these single experiments are representative of seven (a), three (b), three (c), and six (d) separate experiments.

Results

Temporal Characteristics of [^{3}H]cAMP Production. Isoprenaline and salmeterol appear as full agonists of β_2 adrenoceptor-mediated [^{3}H]cAMP production in CHO-β_2 cells (Table 1). Alprenolol and CGP 12177, initially believed to be simply β_2 adrenoceptor antagonists, stimulated small increases in [^{3}H]cAMP that were 3 to 5% of the maximal response to isoprenaline and are therefore very weak agonists. Furthermore, whichever way [^{3}H]cAMP was measured (in the absence or presence of a phosphodiesterase inhibitor, at 10 min, 1 h, or 5 h), alprenolol and CGP 12177 were only ever able to induce maximal responses that were 3 to 5% of the maximal induced by salmeterol and isoprenaline (Table 1).

To determine the time course of cAMP generation, responses to maximum concentrations of ligands were measured at set times from 0 to 5 h in the absence of phosphodiesterase inhibitors. Intracellular and extracellular (secreted/exported) [^{3}H]cAMP levels were measured separately. Isoprenaline (10 μM) stimulated an increase in intracellular [^{3}H]cAMP that was maximal (10.3 ± 2.9-fold over basal, n = 4) within 5 min of the addition of the agonist (Fig. 1a). Salmeterol (100 nM) produced a similar rapid increase (to 96.9 ± 1.1% of the isoprenaline maximum, n = 4).

Measurements of extracellular [^{3}H]cAMP show that [^{3}H]cAMP was secreted from the cells in a time-dependent manner (n = 8) (Fig. 1b). It seemed that the majority of [^{3}H]cAMP was actually secreted. Thus, after 5 h of incubation with 10 μM isoprenaline, the extracellular [^{3}H]cAMP was increased from a mean basal at time 0 of 1792 to a mean of 58,799 dpm ($p < 0.001$, n = 8, paired t test) (Fig. 1b), whereas the basal extracellular [^{3}H]cAMP increased to 1.44 ± 0.26-fold over time 0 basal (n = 4). Salmeterol (100 nM, 82.3 ± 1.3% isoprenaline, 5 h of secretion, n = 4) stimulated a similar secretion of [^{3}H]cAMP.

However, no increase in intracellular [^{3}H]cAMP was seen in response to either alprenolol (10 μM, n = 4) (Fig. 2a) or CGP 12177 (10 μM, n = 4). Despite the lack of intracellular [^{3}H]cAMP, alprenolol and CGP 12177 (both 10 μM) stimulated an increase in exported [^{3}H]cAMP such that the 5-h secreted [^{3}H]cAMP responses were 5.69 ± 0.09% (n = 4) and 6.18 ± 0.28% (n = 4) of the isoprenaline maximum, respectively (Fig. 2b). Significant increases over 5-h basal in secreted [^{3}H]cAMP were also seen after incubation with other agonists ($p < 0.05$, paired t test) (Fig. 3). To further confirm these findings, concentration-responses relationships for agonist-stimulated secretion of extracellular [^{3}H]cAMP were measured after 5 h of incubation in the absence of IBMX. These concentration-response curves were very similar to those seen using the standard cAMP assay (Fig. 4 and Table 1).

Time Required for CRE-Mediated SPAP Production. To determine the length of time required from the addition of agonist until a gene transcription response was measurable, cells were incubated for 10 min to 5 h with the agonist, lysed, and intracellular CRE-SPAP was determined. After 5 h of incubation, isoprenaline stimulated an increase in intracellular SPAP production that was 3.25 ± 0.22-fold over basal (log EC_{50} = −8.70 ± 0.21, n = 8) (Fig. 5a). After 4 h of incubation with isoprenaline, the intracellular SPAP content was 68.5 ± 3.3% that of the 5-h maximum (n = 8). Incubations of 3 and 2 h produced 45.2 ± 3.3 and 21.4 ± 1.8% of the maximum 5-h isoprenaline response, respectively (n = 8) (Fig. 5a). However, 1-h, 30-min, and 10-min incubations with isoprenaline were not sufficient to stimulate any detectable increase in the level of intracellular SPAP (Fig. 5a and Tables 2 and 3). This showed that at least 2 h were needed from the

addition of isoprenaline to the production of a detectable increase in intracellular CRE-SPAP reporter production.

Salbutamol and salmeterol stimulated a pattern of intracellular SPAP production similar to that seen for isoprenaline (Fig. 5b and Tables 2 and 3). The direct adenylyl cyclase stimulator, forskolin, stimulated an intracellular increase in SPAP production over 5 h that was 116.7 ± 4.7% of that of isoprenaline (n = 3) (Fig. 5c and Tables 2 and 3). Also, the relative proportions of the gene transcription signal at 4, 3, and 2 h were similar to those of the full agonists.

The partial agonists labetalol, CGP 12177, and alprenolol (Fig. 5d), however, also initiated a measurable increase in intracellular SPAP within 2 h from the addition of the agonist. Furthermore, the proportions of the responses achieved at 2, 3, and 4-h agonist incubations, compared with the maximum achieved with each ligand over 5 h, were very similar to those achieved with the full agonists (Tables 2 and 3). This suggests that the cAMP threshold that is required to initiate CRE-gene transcription is very small.

Because SPAP is usually measured as a secreted rather than an intracellular reporter, accumulation of SPAP secretion into the surrounding media was assessed. The cells were incubated with agonist for 10 min to 5 h, and the total accumulated SPAP in the surrounding media at that time point was sampled and determined. As expected, the basal SPAP secretion increased over time as more SPAP was secreted into the media. However, the time taken from the addition of agonist until the generation of a measurable response (2 h) and the relative proportions of the responses at 2 to 5 h were very similar to those for intracellular SPAP for all agonists examined (isoprenaline, salbutamol, salmeterol, CGP 12177, and forskolin; data not shown).

Effect of Agonist Removal on CRE-Mediated Gene Transcription. Although the CRE-SPAP measurements above suggest that 2 h was required from the addition of the agonist to achieve a measurable increase in CRE-SPAP production, the agonist was present for the whole incubation. However, the use of a secreted reporter gene, such as SPAP, provides a unique opportunity to assess how long an agonist needs to be present initially to start the CRE-mediated gene transcription process. This is because the agonist can be washed out at set times, and the rate of gene product secretion can be monitored several hours later with a good signal-to-noise ratio. Thus, after agonist removal, the cells were then incubated for a second period of time such that all wells had a total of 5 h of incubation; e.g., after a 10-min agonist incubation, a second incubation was 4 h and 50 min. After the full 5-h incubation, serum-free media were removed from all wells and 300 μl of fresh serum free media added to each well. A 20-μl portion of this media was sampled after a further hour of incubation to give an indication of the rate of gene transcription at 5 to 6 h after different lengths of agonist stimulation times.

Under these conditions, a 5-h isoprenaline incubation stimulated an increase in secreted SPAP production that was 8.0 ± 0.5-fold over basal (n = 7) (Fig. 6a and Table 4). A response was also seen after an initial 10-min isoprenaline stimulation; however, this occurred at a much higher agonist concentration and probably represents the extent to which agonist is retained on the receptor during the wash steps (Fig. 6a and Table 4). The presence of residual agonist in "washed" wells was confirmed in experiments in which media were transferred to fresh cell plates and the SPAP response subsequently measured after 5 h. The isoprenaline response

TABLE 4

Log EC_{50} values for the rate of SPAP secretion between 5 and 6 h after the addition of agonists

The agonists were incubated with the cells for 10 min to 5 h, the agonists were washed out, and the cells were incubated for the remaining time in serum-free media for up to a total of 5 h of incubation. SPAP secretion between 5 and 6 h was then measured. Values represent means ± S.E.M. of n determinations. For isoprenaline and salbutamol, the log EC_{50} values for the two components are shown and the percentage that represented the higher potency site 1 in each case. Only one component was seen with salmeterol and CGP 12177 as agonists.

	Log EC_{50} Site 1	Log EC_{50} Site 2	% Site 1	n
Two-Component Fit				
Isoprenaline				
5 h	−8.45 ± 0.19		100	7
2 h	−8.34 ± 0.15	−4.99 ± 0.34	81.3 ± 3.0	7
1 h	−8.51 ± 0.17	−5.65 ± 0.17	50.8 ± 2.5	9
30 min	−8.25 ± 0.23	−5.56 ± 0.09	26.6 ± 3.2	10
10 min		−6.00 ± 0.12	0	7
Salbutamol				
5 h	−8.60 ± 0.06		100	7
2 h	−8.46 ± 0.04	−5.02 ± 0.29	72.8 ± 5.7	5
1 h	−8.57 ± 0.14	−5.82 ± 0.28	41.2 ± 3.8	6
30 min	−8.67 ± 0.25	−5.81 ± 0.30	17.1 ± 2.8	3
10 min		−5.83 ± 0.26	0	7
One-Component Fit	Log EC_{50}			
Salmeterol				
5 h	−10.74 ± 0.16			3
2 h	−10.61 ± 0.31			3
1 h	−10.53 ± 0.19			3
30 min	−10.43 ± 0.18			3
10 min	−10.10 ± 0.13			3
CGP 12177				
5 h	−9.51 ± 0.14			7
2 h	−8.28 ± 0.18			4
1 h	−9.43 ± 0.09			6
30 min	−9.28 ± 0.03			6
10 min	−8.85 ± 0.09			6

therefore seemed to consist of two components. The responses seen when isoprenaline was present for only the first 30 min, 1 h, and 2 h of the 5-h incubation revealed two component responses that seemed to contain both the high-potency 5-h component and the lower potency 10-min component (Fig. 6a and Table 4). A similar pattern was observed with salbutamol (Fig. 6b and Table 4).

A different pattern was seen with salmeterol and CGP 12177 as agonists. Washing out the drug after 10 min to 2 h had little effect on the gene transcription response compared with the 5-h response (Fig. 6, c and d; Table 4). Salmeterol is known to have a long duration of action because of its ability to bind to an exosite on transmembrane 4 of the β_2 adrenoceptor (Green et al., 1996). The head group then interacts in a competitive manner with the agonist binding site on the β_2 adrenoceptor. CGP 12177 has a long dissociation time of 65 min (Baker et al., 2002). This reporter gene assay therefore provides an excellent tool for examining the "stickiness" of an agonist and demonstrates that a short application of a sticky ligand will stimulate more gene transcription than will short application of a short-acting agonist, even if both stimulate a similar initial increase in intracellular cAMP.

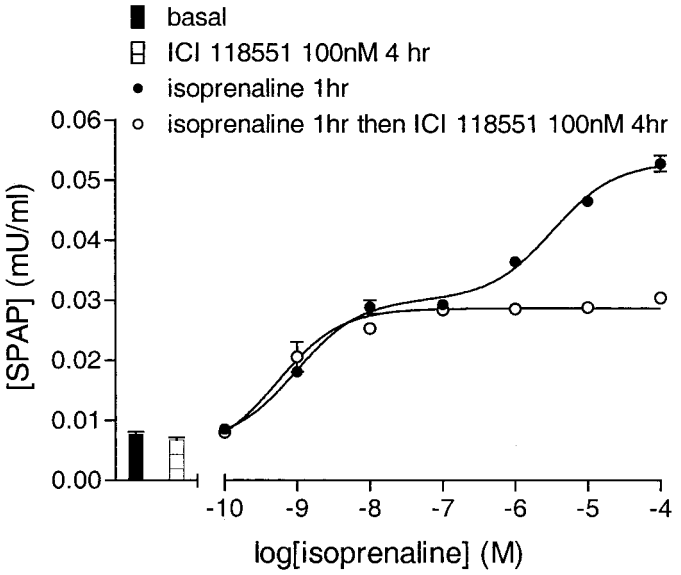

Fig. 7. Rate of CRE-SPAP secretion at 5 to 6 h after the addition of agonist in response to isoprenaline. Isoprenaline was incubated for 1 h and then washed off. The cells were then incubated for a further 4 h in the presence of either serum-free media (●) or 100 nM ICI 118551 (○). The rate of gene transcription between 5 and 6 h was then measured. Data points are mean ± S.E.M. of triplicate determinations from single experiments. Bars represent the basal rate of SPAP secretion and that after 1 h of serum-free media followed by 4 h of 100 nM ICI 118551. This is representative of three separate experiments.

Removing the Agonist from the Receptor by the Addition of an Excess of Sticky Antagonist. To study the effects of short agonist incubations on gene transcription responses without the complication of residual agonist remaining bound to the β_2 adrenoceptors, 100 nM ICI 118551, a sticky antagonist (100 to 1000-fold higher than its K_D value) (Baker et al., 2002) was added to the cells after "washing out" the agonist. This prevented the appearance of a second component (caused by residual agonist) and thus allowed the gene transcription caused by limited agonist times to be studied (Fig. 7). After 10 min of isoprenaline stimulation (i.e., after washout of the agonist and 4 h and 50 min in the presence of 100 nM ICI 118551), no response to isoprenaline was seen (Fig. 8). This suggests that a 10-min stimulation with isoprenaline alone is not sufficient to initiate a gene transcription response. A 30-min isoprenaline incuba-

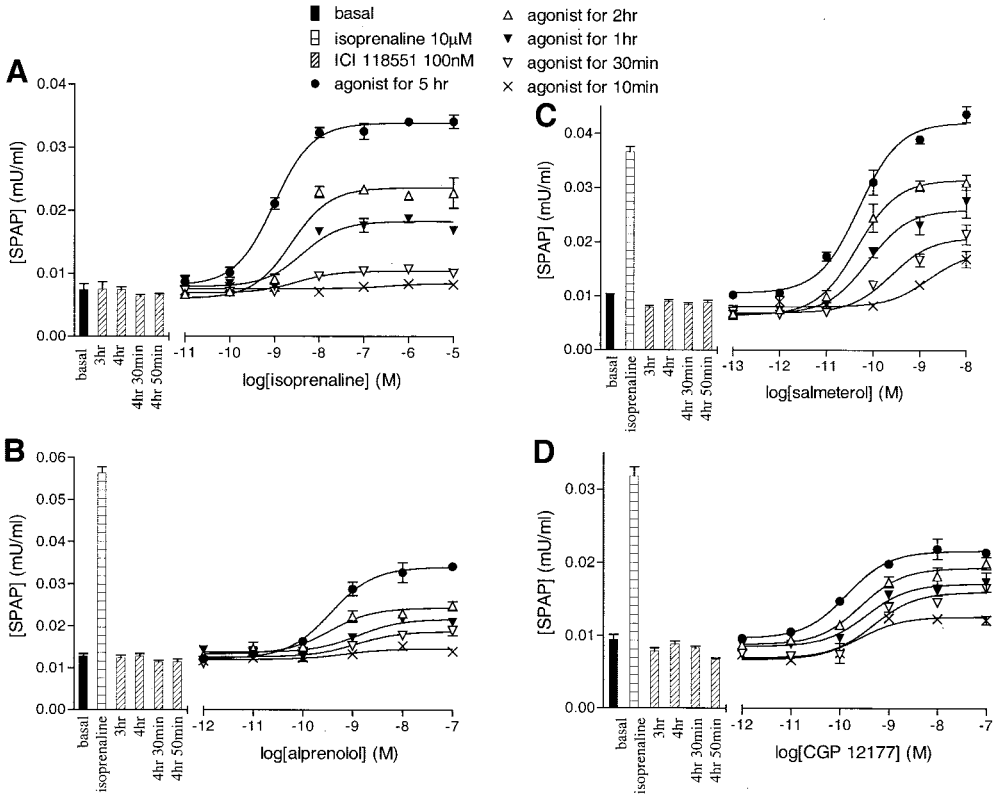

Fig. 8. Rate of CRE-SPAP secretion at 5 to 6 h after the addition of agonist in response to isoprenaline (a), alprenolol (b), salmeterol (c), and CGP 12177 (d). The agonist was added for 10 min to 5 h and then washed away, and the cells were incubated for the remainder of the 5 h in the presence of 100 nM ICI 118551. The rate of SPAP secretion between 5 and 6 h was then measured. Bars represent the basal rate of SPAP secretion, the rate after 5 h of incubation with 10 μM isoprenaline, and the rate after 3 h to 4 h and 50 min with 100 nM ICI 118551. Data points are mean ± S.E.M. of triplicate determinations and these single experiments are representative of seven (a), three (b), four (c), and four (d) separate experiments.

tion, before terminating the isoprenaline-receptor interaction, gave an increase of SPAP production that was 25.6 ± 2.0% of the maximal 5-h response (n = 11) (Fig. 8a and Tables 5 and 6). Progressive increases in the SPAP production were seen with longer incubation times (Fig. 8a and Tables 5 and 6). These proportions are very similar to the proportions of the high-potency component (component 1 of Table 4 and Fig. 6) seen after the drug washout mentioned above. The gene transcription responses to the partial agonist alprenolol were similarly terminated by the addition of 100 nM ICI 118551, and a similar profile of gene transcription was obtained (Fig. 8b and Tables 5 and 6).

After the removal of the extracellular agonist and the addition of ICI 118551, the responses to the long-acting agonists salmeterol and CGP 12177 were also reduced (Fig. 8, c and d; Tables 5 and 6). However, sufficient agonist was still on the receptor to induce an agonist response even if the ICI 118551 was added after only 10 min. With salmeterol, which would remain tethered to the receptor and just the head group in the binding pocket compete with ICI 118551, as the maximum reduces (30 and 10 min), the curve becomes right-shifted, suggesting competitive antagonism of the interaction of the salmeterol head group with the active site of the β_2 adrenoceptor.

Stimulation of Ser133 Phosphorylation of CREB. Stimulation of nuclear CREB phosphorylation by protein kinase A is the key event in cAMP-mediated activated of gene transcription (Mayr and Montminy, 2001). In CHO-β_2 SPAP cells, both isoprenaline (10 μM) and alprenolol (10 μM) produced a rapid (within 10 min) stimulation of phospho-CREB accumulation, which was maintained for up to 5 h (isoprenaline, n = 8: alprenolol, n = 4) (Fig. 9). The ubiquitous CREB family member activated transcription factor 1 was also phosphorylated in response to both agents with a similar time course (Fig. 9).

c-fos Gene Transcription. All of the experiments described above are using a stable cell line expressing a synthetic promoter (6 CRE elements) upstream of a reporter protein (SPAP). To determine whether β_2 agonists could stimulate gene transcription via a natural promoter, a mixed population of CHO-β_2 cells, secondarily transfected with the human c-fos promoter (which contains a CRE) (Hill et al., 2001) upstream of a luciferase construct were used. Here, isoprenaline stimulated an increase in c-fos–luciferase production (log EC_{50} = −8.50 ± 0.16, 1.5 ± 0.07-fold over basal, n = 8) (Fig. 10a). The direct adenylyl cyclase stimulator forskolin also stimulated an increase in c-fos–luciferase (log EC_{50} = −6.50 ± 0.09, n = 4) (Fig. 10b), which is very similar to that reported for cAMP production and the rate of CRE-SPAP gene transcription in CHO cells (Baker et al., 2003b) and intracellular SPAP (Fig. 5c and Tables 2 and 3). Alprenolol and CGP 12177 were also able to stimulate gene transcription via this natural promoter (alprenolol: log EC_{50} = −9.27 ± 0.24, 39.6 ± 3.0% of isoprenaline maximum, n = 4; CGP 12177: log EC_{50} = −9.15 ± 0.33, 45.1 ± 4.0% of isoprenaline maximum, n = 5) (Fig. 10, c and d). Thus, the stimulation of luciferase via a natural CRE-containing promoter (c-fos) is very similar to the stimulation of the CRE-SPAP production above.

The Expression of an Endogenous Protein in CHO Cells Expressing the Human β_2 Adrenoceptor. Finally, the ability of β ligands to stimulate an endogenous protein was examined. Isoprenaline (10 μM) and forskolin (10 μM) produced a transient increase in the expression of c-fos (band running at approximately 55 kDa) between 30 min and 2 h of stimulation in CHO-β2-SPAP cells. This was detected with two different c-fos antibodies (pan c-fos–K25; n = 7 for isoprenaline, n = 4 for forskolin; and selective c-fos-4, n = 4 for isoprenaline) (Fig. 11). A similar pattern of expression of c-fos protein has been observed in CHO cells in response to insulin (Griffiths et al., 1998). The pan c-fos antibody K25 also detects FRA-1 (Griffiths et al., 1998), and several studies have shown that cAMP analogs and forskolin can increase the abundance of FRA-1 (Liu et al., 1995; Li et al., 1997). Isoprenaline produced a sustained increase in the expression of FRA-1 expression in CHO-β_2-SPAP cells that was detectable after 1-h agonist stimulation and maintained for up to 5 h (the longest time point measured). This was seen as an increase in intensity of a triplet of bands of apparent molecular mass 35 to 40 kDa, which has been shown previously to represent FRA-1 in CHO cells (Griffiths et al., 1998) and which was detected with both the pan c-fos–K25 antibody (n = 8) and the FRA-1 specific antibody (n = 3) (Fig. 12). Forskolin, alprenolol, and CGP 12177 also stimulated a sustained increase in endogenous FRA-1 expression, detected again by both antibodies (forskolin: c-fos-K25, n = 4 and FRA-1, n = 4; alprenolol: c-fos-K25, n = 6 and FRA-1, n = 3; CGP 12177: c-fos-K25, n = 4 and FRA-1, n = 4) (Fig. 12).

Discussion

Many clinically used drugs are GPCR antagonists and are given to prevent receptor activation by endogenous agonists. In other conditions, their mode of action is unclear (e.g., β-blockers in heart failure). Most GPCR antagonist drugs are administered daily, aiming to achieve 24-h receptor blockade, and are believed to have little efficacy of their own. However, recent studies show that many clinically used "antagonists"

TABLE 5

Percentage maximum responses for the rate of SPAP secretion between 5 and 6 h after the addition of agonists

The agonists were incubated with the cells for 10 min to 5 h, the agonists were washed out, and 100 nM ICI 118551 was incubated for the remaining time up to a total of 5 h of incubation. SPAP secretion between 5 and 6 h was then measured. Values represent means ± S.E.M. of n determinations.

	% Isoprenaline		% of Maximal Agonist Response											
Agonist	5 h	n	4 h	n	3 h	n	2 h	n	1 h	n	30 min	n	10 min	n
Isoprenaline	100	10	92.3 ± 9.4	3	93.3 ± 11.6	3	73.4 ± 6.1	7	44.5 ± 1.5	10	25.6 ± 2.0	11	N.R.	10
Salmeterol	111.5 ± 3.6	4	101.8 ± 10.8	4	89.1 ± 8.8	4	85.6 ± 7.8	4	64.3 ± 0.2	4	54.4 ± 3.8	4	52.1 ± 8.0	4
Alprenolol	53.2 ± 6.6	4	80.8 ± 14.3	4	82.8 ± 6.7	4	61.4 ± 5.1	4	41.0 ± 1.7	4	25.7 ± 3.2	4	13.1 ± 2.7	3
CGP 12177	52.8 ± 3.5	4	95.0 ± 11.8	4	100.8 ± 9.5	4	91.9 ± 3.8	4	75.4 ± 5.1	4	48.6 ± 9.5	4	36.4 ± 5.3	4

% isoprenaline, maximum 5-h response of each ligand expressed as a percentage of the maximum 5-h response to 10 μM isoprenaline as determined in each experiment; % of maximal agonist response, response of that ligand expressed as a percentage of the maximum 5-h response for that same ligand; N.R., no response seen.

TABLE 6
Log EC_{50} values for the rate of SPAP secretion between 5 and 6 h after the addition of agonists
The agonists were incubated with the cells for 10 min to 5 h, the agonists were washed out, and 100 nM ICI 118551 was incubated for the remaining time up to a total of 5 h of incubation. SPAP secretion between 5 and 6 h was then measured. Values represent means ± S.E.M. of *n* determinations.

Agonist	Log EC_{50} Values															
	5 h	*n*	4 h	*n*	3 h	*n*	2 h	*n*	1 h	*n*	30 min	*n*	10 min	*n*
Isoprenaline	−8.20 ± 0.21	10	−8.82 ± 0.22	3	−8.74 ± 0.16	3	−8.25 ± 0.20	7	−8.39 ± 0.21	10	−8.26 ± 0.17	11	N.R.	10
Salmeterol	−10.63 ± 0.13	4	−10.54 ± 0.08	4	−10.55 ± 0.34	4	−10.35 ± 0.04	4	−10.08 ± 0.09	4	−9.36 ± 0.18	4	−8.54 ± 0.17	4
Alprenolol	−9.36 ± 0.07	4	−9.53 ± 0.04	4	−9.40 ± 0.13	4	−9.38 ± 0.07	4	−9.17 ± 0.12	4	−9.08 ± 0.30	4	−9.51 ± 0.23	3
CGP 12177	−9.65 ± 0.09	4	−9.45 ± 0.13	4	−9.67 ± 0.05	4	−9.56 ± 0.06	4	−9.35 ± 0.15	4	−9.40 ± 0.05	4	−9.35 ± 0.10	4

N.R., no response seen.

actually have low efficacy, either positive or negative (Chidiac et al., 1994; Azzi et al., 2001; Smit et al., 1996; Baker et al., 2003a,b). This study suggests that antagonists with little efficacy at the secondary messenger level can stimulate significant downstream events (e.g., changes in gene transcription).

Large increases in intracellular [^{3}H]cAMP occurred within 1 min of isoprenaline addition to cells but declined after approximately 90 min. Potential reasons include isoprenaline-induced receptor desensitization, depletion of intracellular substrate ([^{3}H]adenine), induction of endogenous phosphodiesterases, and [^{3}H]cAMP secretion from the cells. Isoprenaline stimulates PKA and β-adrenergic receptor kinase-mediated receptor phosphorylation (Clark et al., 1999), uncoupling the receptor G-protein complex, and reducing agonist affinity (Yuan et al., 1994). The receptor then internalizes via clathrin-coated pits (Krupnick and Benovic, 1998). The decrease in intracellular [^{3}H]cAMP over time could be caused by receptor desensitization and internalization. However, salmeterol, a less efficient agonist stimulating little β-adrenergic receptor kinase phosphorylation and receptor internalization (January et al., 1998), stimulated a similar pattern of [^{3}H]cAMP production.

[^{3}H]cAMP was secreted from cells over time, and this export continued even when intracellular concentrations of [^{3}H]cAMP declined, making the depletion of substrate ([^{3}H]adenine) an unlikely explanation for the decrease in intracellular [^{3}H]cAMP. The increase and decrease of intracellular cAMP stimulated by isoprenaline and salmeterol was very similar to that reported previously (Florio et al., 1999; Godhinho and Costa, 2003). These authors also showed that forskolin stimulated a similar pattern, confirming that the intracellular decline in cAMP is not caused by receptor desensitization/internalization. Similar results were also obtained with concentrations of IBMX that had totally suppressed cell extract cAMP degradation, leading the authors to suggest that the decline is not caused by intracellular phosphodiesterase induction (Godhinho and Costa, 2003) but by cAMP export. Others, however, have shown an up-regulation of phosphodiesterase activity after an increase in intracellular cAMP (Chern et al., 1993; Le Jeune et al., 2002).

The isoprenaline and salmeterol-induced cAMP export was also very similar to that described previously (Finnegan and Carey, 1998; Florio et al., 1999; Godhinho and Costa, 2003). However, in these cases, a substantial increase in intracellular cAMP was also seen, leading the authors to conclude that cAMP efflux is proportional to intracellular generation of cAMP. Here, we have shown that alprenolol and CGP 2177

Fig. 9. Western blots showing the detection of phosphorylated (phospho-) CREB and phosphorylated activated transcription factor 1 under basal conditions and after stimulation with isoprenaline (10 μM) or alprenolol (10 μM). This single experiment is representative of eight (isoprenaline), four (alprenolol), and four (basal) separate experiments.

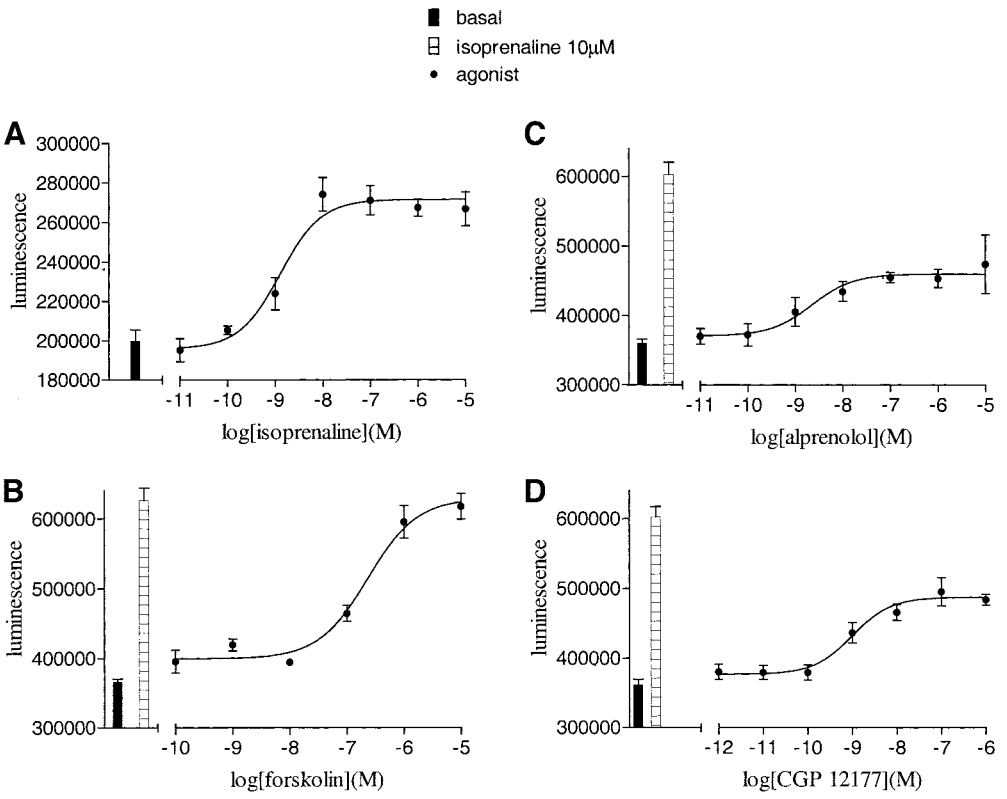

Fig. 10. Production of luciferase in response to isoprenaline (a), forskolin (b), alprenolol (c), and CGP 12177 (d) in a mixed population of CHO-β_2–c-fos–luciferase cells in which the luciferase production is under the control of a natural c-fos promoter. Data points are mean ± S.E.M. of triplicate determinations. Bars represent the basal luciferase production and that in response to 10 μM isoprenaline. These single experiments are representative of eight (a), four (b), four (c), and five (d) separate experiments.

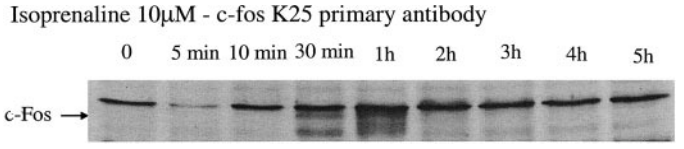

Fig. 11. Western blot showing the detection of endogenous c-fos (55-kDa band; c-fos K25 antibody) under basal conditions and at various times after the addition of 10 μM isoprenaline. The arrow represents the location of the c-fos protein at 55 kDa. This single experiment is representative of eight separate experiments.

did not stimulate any measurable increase in intracellular [^{3}H]cAMP at any stage. However, they clearly increased [^{3}H]cAMP production as they stimulated a small export of [^{3}H]cAMP. It therefore seems that [^{3}H]cAMP export reflects intracellular turnover of [^{3}H]cAMP rather than absolute concentration.

After the large stimulation of intracellular [^{3}H]cAMP, isoprenaline stimulated a large increase in CRE-mediated SPAP expression. Two hours was required from the addition of isoprenaline for the generation of a measurable increase in the SPAP protein, probably reflecting the time taken for transcription, translation, and assembly of the active protein. Thereafter, the longer the incubation, the more protein product generated. A similar pattern was seen with other full agonists.

Weak agonists stimulating immeasurable quantities of intracellular [^{3}H]cAMP might be expected to behave differently. If an intracellular cAMP threshold was required to initiate CRE-gene transcription (e.g., threshold was half-way along the y-axis of Fig. 13), weak partial agonists might not stimulate gene transcription at all. Alternatively, if a total quantity of cAMP was needed to initiate CRE-gene transcription (i.e., area under the curve, Fig. 13), then weak partial agonists would be slower to initiate the process.

Alprenolol and CGP 12177 clearly stimulated CRE-gene transcription even though no increase in intracellular [^{3}H]cAMP was detectable. It therefore seems unlikely that a significant concentration threshold of cAMP needs to be reached to initiate CRE-gene transcription. The increase in CRE-gene transcription induced by the partial agonists was also detectable 2 h after the addition of these ligands. Furthermore, the gene transcription product increased proportionally over time in the same manner as that seen with the full agonists and the direct adenylyl cyclase stimulator forskolin.

A further indicator that overall intracellular cAMP concentration or total quantity is less important comes from an examination of the log EC_{50} values for CRE-gene transcription at different times. At shorter incubation times, lower concentrations of agonist would generate smaller amounts of intracellular cAMP, which might not exceed the threshold required for the initiation of CRE-gene transcription and so not result in a response. Thus, if cAMP concentration or total quantity determined the initiation of CRE-gene transcription (achieved by either a shorter incubation of a larger concentration of agonist or a longer incubation of a lower concentration of agonist), the curves would be expected to become progressively left-shifted over time. However, the EC_{50} values are similar for both the 5-h and the 2-h responses. This was equally true for partial agonist responses. The absolute intracellular cAMP concentration and quantity required to initiate CRE-gene transcription is therefore very low and is lower than that for detection of whole-cell intracellular [^{3}H]cAMP. This observation is in keeping with previous reports in which partial agonists achieve substantial PKA activation (Yuan et al., 1994; January et al., 1998). However, cAMP levels within localized areas of cells may become sub-

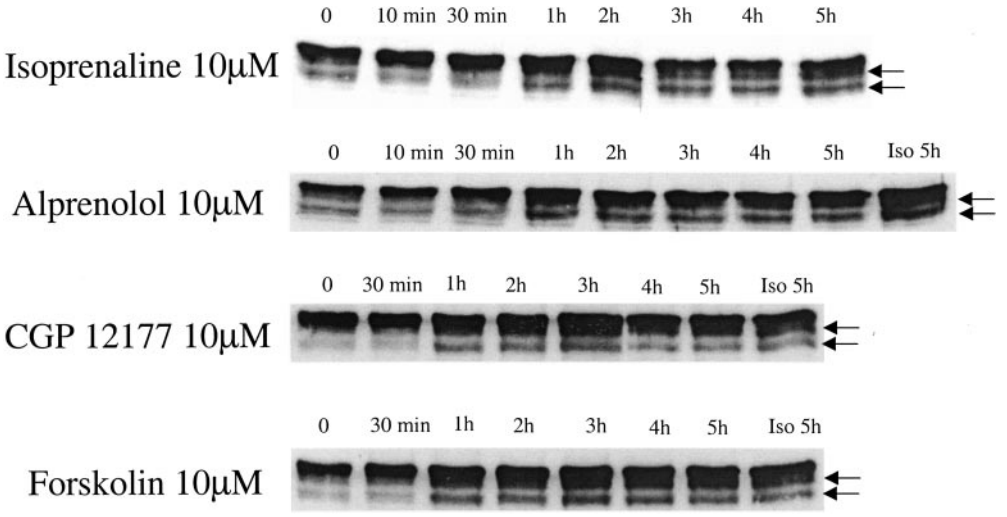

Fig. 12. Western blots showing the detection of the endogenous protein FRA-1 over time in CHO-β2-SPAP cells using an FRA-1 specific antibody. The two FRA-1 (35–40 kDa) bands are seen immediately below the nonspecific band visible in all lanes in response to isoprenaline (a), forskolin (b), alprenolol (c), and CGP 12177 (d). The last lane of each blot shows the response to isoprenaline 10 μM after 5 h. These single experiments are representative of three (a), four (b), three (c), and three (d) separate experiments. Similar increases in FRA-1 were seen using the pan c-fos antibody K25.

stantially increased but not detectable when examining whole-cell [^{3}H]cAMP levels (Insel, 2003).

Finally, the length of time of agonist exposure required to initiate CRE-gene transcription was investigated. A 10-min incubation with isoprenaline did not stimulate gene transcription. Thus, although maximal concentrations (and large quantities) of intracellular cAMP were stimulated, these were not sufficient to initiate CRE-gene transcription (Fig. 13). An initial isoprenaline incubation of 30-min was required to detect an increase in CRE-gene transcription. Although the cAMP concentration and total quantity generated in these 30 min was very large, the gene transcription response was small. However, the longer the agonist exposure, and hence the longer the cAMP turnover, the larger the CRE-gene transcription response generated. This suggests that the time of cAMP turnover is important in initiating CRE-gene transcription.

A similar pattern was seen with the partial agonists. These also stimulated small gene transcription responses after 30-min incubations. The intracellular cAMP concentration and total quantity produced after 10-min incubations with isoprenaline were far higher than that produced after 1 h with alprenolol (Fig. 13), and yet the 1-h alprenolol exposure induced CRE-gene transcription, whereas the 10 min of isoprenaline did not. This therefore confirms that it is not the absolute cAMP concentration or total quantity that is important in driving CRE-gene transcription but rather the length of time of cAMP turnover.

A major rate-limiting step in cAMP-mediated gene transcription is the nuclear entry of the active catalytic unit of PKA (Hagiwara et al., 1993; Mayr and Montminy, 2001). cAMP levels greater than that required to maintain PKA nuclear entry will therefore not translate into increased signal, and partial agonists should produce a substantial increase in CREB phosphorylation. This was observed in Fig. 9 and adds further support to the hypothesis that the duration of cAMP-mediated CREB phosphorylation is a major determinant of CRE-mediated gene transcription.

The induction of gene transcription via a natural promoter was also seen in response to the partial agonists alprenolol and CGP 12177 (Fig. 10). Finally, the induction of FRA-1 expression in response to isoprenaline, forskolin, alprenolol, and CGP 12177 also suggests that partial agonists can in-

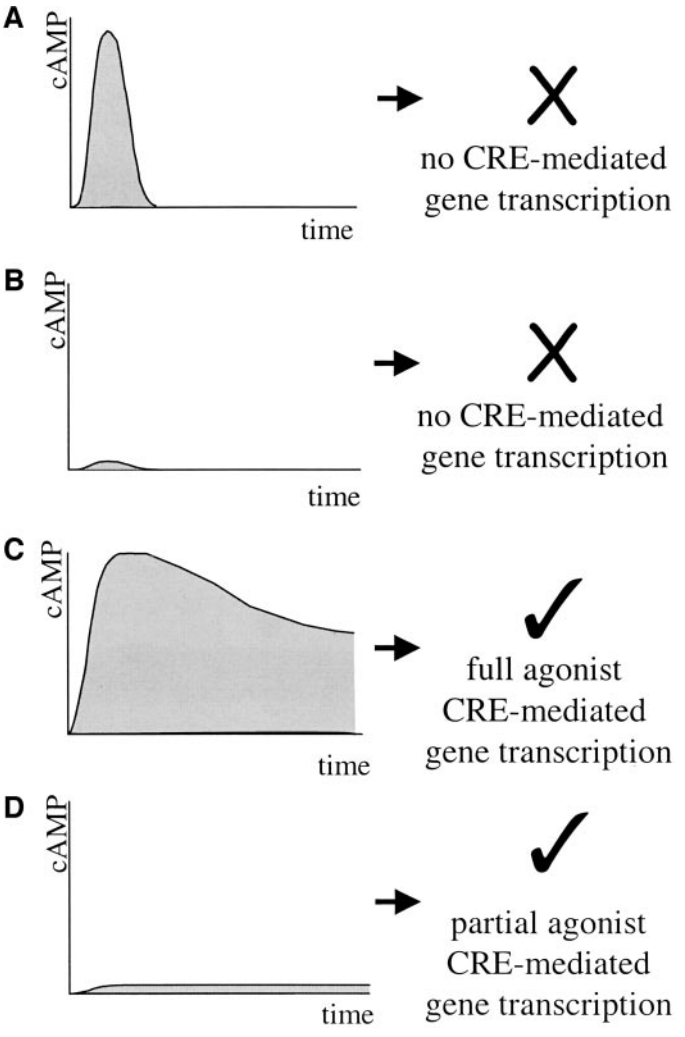

Fig. 13. Schematic representation of the different pattern of intracellular cAMP generation and the subsequent downstream CRE-mediated gene transcription responses. a, 10-min incubation of a full agonist (e.g., isoprenaline). b, 10-min incubation of a weak partial agonist (e.g., alprenolol). c, a longer incubation with a full agonist (e.g., a 5-h isoprenaline incubation). d, a longer incubation with a weak partial agonist (e.g., a 5-h alprenolol incubation).

duce gene transcription of endogenous genes. FRA-1 expression increased in response to all agonists 1 h after incubation with agonist. There was therefore no delay in the initiation of gene transcription by lower efficacy agonists, again making it unlikely that a total quantity of intracellular cAMP is required to initiate the process.

In conclusion, this study suggests that the major driving force in the generation of CRE-gene transcription is not the concentration or total quantity of cAMP generated but rather a sustained turnover of intracellular cAMP. The actual concentration of intracellular cAMP required to initiate CRE-gene transcription is exceedingly small and may be lower than the level of detection. Furthermore, many drugs classified previously as antagonists (based on second-messenger assays) may actually achieve this. Because these drugs are administered to achieve 24-h receptor occupancy, these effects may result in previously unrecognized but substantial gene transcription. This may be especially relevant in conditions in which their mode of action is not obvious (e.g., β-blockers in heart failure). Furthermore, gene transcription responses caused by long-term antagonists may be far more substantial than any caused by very effective but rapidly removed endogenous agonists (e.g., adrenaline).

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