RESEARCH COMMUNICATION

Evaluation of the use of the luciferase-reporter-gene system for gene-regulation studies involving cyclic AMP-elevating agents

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The effects of cyclic AMP (cAMP)-elevating agents on the activity of *cis*-acting gene promoter sequences are frequently studied using the luciferase-reporter-gene system. The aim of the present study was to assess whether cAMP-elevating agents induce any changes in the level of luciferase activity independently of a transcriptional activation of promoter elements. Chloramphenicol acetyltransferase (CAT) and luciferase reporter genes under the control of the same promoter elements were transiently expressed in primary cultures of human vascular smooth-muscle

cells. Transfected cells were treated with a cell-permeable and non-hydrolysable cAMP analogue, 2'-O-monobutyl-8-bromo cAMP, or with the cAMP-elevating agents forskolin and prostaglandin E₁ (PGE₁). Forskolin and PGE₁ induced a significant increase in the level of luciferase activity, but had no effect on CAT activity. Conclusions based solely on the use of the luciferase-reporter-gene system in studies involving promoter activation by cAMP-elevating agents could therefore be misleading.

INTRODUCTION

The regulation of eukaryotic gene transcription is mediated by *cis*-acting sequence motifs which are typically, although not exclusively, located within the 5'-flanking region of genes [1]. The *in vitro* study of transcriptional regulation commonly involves the transfection of cultured cells with reporter-gene constructs. These constructs comprise the putative regulatory sequences under investigation linked to a downstream reporter gene [2]. The transcriptional effects of potential inducers or repressors of gene expression are usually assessed by treating the transfected cells with chemical or biological agents prior to preparation of the transfected cell lysates and measurement of reporter protein activity. The most common reporter-gene systems, such as chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (*neo*), and firefly luciferase (LUC) fulfil the following requirements: the activity of the encoded protein is easy to assay and is either absent from non-transfected cells or present only at very low levels [3,4].

Firefly LUC offers several advantages over the other reporter proteins: the assay for LUC activity is more sensitive and easier to use than the assay for CAT activity [5,6]. In addition, LUC is a monomeric protein which does not require post-translational modification and hence its activity can be assessed immediately upon translation [7,8]. The LUC reaction emits light in the visible spectrum (560 nm) through mono-oxygenation of luciferin with O₂ and ATP as co-substrates according to the reaction [9]:

In the *in vitro* LUC reaction assay, saturating concentrations of luciferin, ATP and Mg²⁺ should be used so that they are not rate-limiting. The emission of light is therefore proportional to the amount of LUC present, which therefore correlates with the level of reporter-gene expression. The luciferin–LUC system is one of

the most sensitive assays for the direct measurement of ATP and hence for the indirect measurement of ADP, AMP and cyclic AMP (cAMP) [9]. This luminescence assay of adenosine phosphate molecules is based on their conversion into ATP and the subsequent determination of ATP by its luminescent reaction with firefly luciferin and LUC following the reaction described above.

It is well established that cAMP plays an important role in many cellular processes through its action as second messenger, transmitting the biological effects of various hormones and growth factors [10,11]. The downstream targets of this messenger are cAMP-responsive elements present in the regulatory regions of many genes [12]. The regulatory effects of cAMP-elevating agents on various gene promoters are frequently studied using the LUC-reporter-gene system [13–20]. Bearing in mind the sensitivity of firefly LUC to adenosine phosphate molecules, the measured emission of light may not necessarily reflect the level of LUC generated from the reporter-gene construct. The aim of the present study was to assess whether or not cAMP would interfere with the activity of transiently expressed LUC. It is demonstrated here that treatment of cells with a cAMP-elevating agent could result in a substantial increase in the level of measurable LUC activity. The use of this reporter-gene system in studies involving promoter regulation by cAMP-elevating agents could therefore lead to misleading conclusions.

MATERIALS AND METHODS

Materials

Anti-(human smooth-muscle cell α -actin) monoclonal antibody, 3-isobutyl-1-methylxanthine (IBMX), forskolin, prostaglandin E₁ (PGE₁), 2'-O-monobutyl-8-bromo cyclic AMP (Bu-8-Br-cAMP), dibutyl cAMP, sodium salt AMP, ADP, ATP and cAMP were purchased from Sigma. The cAMP ¹²⁵I-radioimmunoassay kit was purchased from Amersham Inter-

Abbreviations used: HVMSC, human vascular smooth-muscle cells; FCS, foetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine; PGE₁, prostaglandin E₁; cAMP, cyclic AMP; Bu-8-Br-cAMP, 2'-O-monobutyl-8-bromo cyclic AMP; CAT, chloramphenicol acetyltransferase; LUC, luciferase.

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national. Transfectam reagent (liposomes: cationic lipopolyamine molecule), reporter lysis buffer, LUC-reporter-gene vectors (pGL2-Control DNA), CAT reporter gene vectors (pCAT-control DNA), pSV- β -galactosidase-reporter-gene vectors, LUC and β -galactosidase enzyme assay were from Promega (Southampton, U.K.). Pure firefly LUC was obtained from Boehringer Mannheim.

Cell culture

Human vascular smooth-muscle cells (HVSMC) were isolated from sections of normal abdominal aorta obtained from kidney transplant donors (10–45 years of age) as described previously [21]. Cells were subcultured at a split ratio of 1:4 and used between passages 2 and 8. HVSMC were identified as such by their characteristic 'hills-and-valleys' pattern of growth and by their positive immunostaining with an anti-(human smooth-muscle cell α -actin) antibody [22].

Transfections, CAT and LUC assays

Transient expression of CAT and LUC constructs in HVSMC was achieved after transfection by liposomes [23,24]. At 1 day prior to transfections, HVSMC were plated at a density of $1.5 \times 10^5/\text{cm}^2$ in Dulbecco's modified Eagle's medium (DMEM)/10% FCS. On the day of transfection, cells were incubated in serum-free medium for 12 h with liposomes/DNA containing 1 μg of pSV- β -galactosidase control plasmid in addition to 1 μg of LUC-positive control (pGL2-control DNA) or CAT-positive (pCAT-control DNA). At 48 h post-transfection, cells were treated for 1 h with 10 μM forskolin, PGE₁, Bu-8-Br-cAMP or diluent. When the cells were treated with IBMX (10 μM), this was added 5 min before the start of the treatment. At the end of the assay period, cell extracts were prepared. CAT activity was determined by liquid-scintillation counting [25], and LUC activity was determined by measuring its activity in 20 μl of cell extract with 100 μl of LUC assay reagent on a model 20e Turner Designs (Sunnyvale, CA, U.S.A.) luminometer. The values for CAT and LUC activity were corrected for transfection efficiency using β -galactosidase activity [26].

cAMP measurement

Parallel cultures of HVSMC pre-treated for 5 min with IBMX (10 μM) were incubated for 1 h in the presence of forskolin (10 μM), PGE₁ (10 μM) or diluent. The cells were harvested by scraping in PBS, collected by centrifugation and treated with 95% ethanol for 30 min on ice. Samples were then centrifuged, and the supernatant collected, dried under vacuum and used for the measurement of cAMP. The pellets were lysed in 0.1 M NaOH and used for the measurement of protein concentration using the Pierce bicinchoninic acid (BCA) kit. cAMP levels were measured using an ¹²⁵I-radioimmunoassay kit according to the manufacturer's (Amersham International) instructions.

RESULTS AND DISCUSSION

LUC and CAT reporter-gene constructs under the control of the same regulatory elements (simian-virus-40 promoter and enhancer elements) were transiently expressed in primary cultures of HVSMC. At 48 h post-transfection, cells were treated for 1 h with 10 μM of cAMP-elevating agents forskolin or PGE₁, or 10 μM Bu-8-Br-cAMP, which is a cell-permeable and non-hydrolysable cAMP analogue [10,11]. Bu-8-Br-cAMP mimics some of the biological effects of cAMP which do not involve the hydrolysis of cAMP. The above treatments were performed in the presence or absence of 10 μM IBMX, a potent inhibitor of

Table 1 Effect of forskolin, PGE₁ and Bu-8-Br-cAMP on LUC and CAT activity

HVSMC were transfected with liposomes/DNA of pSV β -galactosidase control plasmid in addition to either luciferase-positive-control or CAT-positive-control plasmids. At 48 h post-transfection, cells were treated for 1 h with 10 μM of either forskolin, PGE₁, Bu-8-Br-cAMP or diluent in the presence or absence of 10 μM IBMX. Cell extracts were prepared, and β -galactosidase, LUC and CAT activities were determined. Values for CAT and LUC activities were both corrected for transfection efficiency using β -galactosidase activity. Values are means $\pm$ S.E.M. of three independent determinations.

Treatment	10 ⁻³ $\times$ Activity (c.p.m.)			
	CAT		LUC	
	-IBMX	+IBMX	-IBMX	+IBMX
Diluent control	147.6 $\pm$ 19.6	141.0 $\pm$ 6.5	3.60 $\pm$ 0.40	1.27 $\pm$ 0.29
Forskolin	133.6 $\pm$ 7.9	123.6 $\pm$ 24.6	16.83 $\pm$ 1.95	4.07 $\pm$ 0.73
PGE ₁	151.3 $\pm$ 28.7	153.3 $\pm$ 29.8	12.67 $\pm$ 1.98	3.87 $\pm$ 0.43
Bu-8-Br-cAMP	136.6 $\pm$ 8.7	119.3 $\pm$ 9.88	3.87 $\pm$ 0.61	1.30 $\pm$ 0.40

phosphodiesterases, enzymes which hydrolyse cAMP to AMP [10,11]. Measurement of cAMP levels in cultured HVSMC confirmed that forskolin and PGE₁ used at 10 μM for 1 h induced 5.2- and 4.8-fold increases respectively in the cellular levels of cAMP. It should be noted here that, in the present study, dibutyryl-cAMP, another cell-permeable cAMP analogue, was used. Since dibutyryl-cAMP and Bu-8-Br-cAMP gave rigorously similar results, only the data obtained with Bu-8-Br-cAMP are presented.

Table 1 depicts the changes in the levels of CAT and LUC activity in transfected HVSMC treated with the various agents described above. In both the presence or absence of IBMX, neither agent induced any significant change in the level of CAT activity as compared with cells treated with diluent. The level of LUC activity, however, showed a marked change as a result of treatment with forskolin and PGE₁. In the absence of IBMX, treatment of the cells with forskolin or PGE₁ resulted in 4.7- and 3.5-fold increases in LUC activity respectively over that of diluent-treated cells. The LUC activity of cells treated with Bu-8-Br-cAMP was similar to that of diluent-treated cells. Pre-incubation of cells with IBMX reduced the level of LUC activity in both diluent-treated cells and cells treated with forskolin, PGE₁ or Bu-8-Br-cAMP. However, forskolin and PGE₁ induced a 3.2- and 3.0-fold increase in the level of LUC activity as compared with diluent-treated cells. The LUC activity of Bu-8-Br-cAMP-treated cells was similar to that of diluent-treated cells. Using a range of concentrations of pure CAT, LUC and β -galactosidase enzymes, it was verified that the assays for these three reporter-gene activities were performed within a linear range. In the experiments described above, both LUC and CAT reporter genes were placed under the control of the same promoter elements. Since IBMX, forskolin and PGE₁ induced a significant change in the level of LUC activity but had no detectable effect on the level of CAT activity, we may infer that these agents did not affect the extent rate of LUC gene transcription, but rather interfered with the expressed LUC protein activity.

Incubation of transfected cells with the cell-permeable and non-hydrolysable cAMP analogue Bu-8-Br-cAMP did not affect the level of LUC activity. IBMX, which inhibits cellular phosphodiesterases (and hence the conversion of cAMP into AMP, ADP and ATP), reduced both the steady-state level of LUC activity and the increase in LUC activity induced by the cAMP-elevating

Figure 1 Effect of cAMP, AMP, ADP, ATP on LUC activity

Increasing amounts of either cAMP ($\blacktriangle$), AMP ($\blacksquare$), ADP ($\circ$) or ATP ($\square$) were added to either (a) extracts prepared from untreated cells transfected with the LUC reporter gene or 50 pg/ml of pure LUC (b), and LUC activity was determined. Values are means for three independent determinations. The S.E.M. values were less than 5% and are therefore not shown. For Bu-8-Br-cAMP the curves obtained were identical with that of cAMP and are also not shown.

agents forskolin and PGE_1 . Taken together, these data indicate that the observed changes in the level of LUC activity subsequent to forskolin or PGE_1 treatment are not due to cAMP itself.

In order to determine if cAMP derivative molecules mediate the observed increases in LUC activity, increasing amounts of cAMP, Bu-8-Br-cAMP, AMP, ADP or ATP were added to either pure LUC or extracts prepared from HVSMC transfected with the LUC reporter gene and which had not received any further treatment. Whereas AMP and ADP induced a dramatic decrease in the LUC activity of such extracts, neither cAMP, Bu-8-Br-cAMP nor ATP had any significant effect (Figure 1a).

Apart from its insensitivity to changes in the concentration of cAMP and Bu-8-Br-cAMP, pure LUC behaved differently from the LUC present in cell extracts with regard to changes in the concentration of ATP, ADP, and AMP (Figure 1b). ATP induced a drastic decrease in the activity of pure LUC, whereas ADP and AMP had exerted a rather biphasic effect which was more pronounced for AMP. These data are of particular interest, as they underline the extreme sensitivity of the luminescence reaction to variations in the levels of the cAMP derivative molecules AMP, ADP and ATP. cAMP and Bu-8-Br-cAMP are cyclic nucleotides and do not appear to interfere directly with the LUC reaction. The discrepancies observed between the sensitivity of pure LUC and LUC present in the cell extract to changes in the concentrations of ATP, ADP and AMP strongly suggest that these molecules modulate the activity of the LUC protein and that other as-yet-unidentified compounds, present in cell extracts, interfere with their action. The aim of the experiments described above was to determine whether the observed changes in the LUC activity subsequent to treatment of the cells with cAMP-elevating agents could be attributed to a specific molecule. If that were the case, the conclusion of the present study would have been that the LUC-reporter-gene system could still be used in studies involving treatment of cells with cAMP-elevating agents provided that the cell extracts were normalized with regard to the concentration of the interfering agent. The data presented here demonstrate clearly that the observed increases in LUC activity subsequent to treatment of cells with cAMP-elevating agents do not appear to be mediated solely by variations in the cellular levels of specific compounds, but are rather likely to be a result

of complex changes in the levels of multiple compounds. Therefore the normalization of cell extracts with regard to ATP, ADP and AMP cannot be an appropriate solution.

The present study was conducted with HVSMC as an example of primary cell culture. Similar if not identical results were obtained with an established human hepatocyte cell line (HepG 2). Additionally, using forskolin as a cAMP-elevating agent, similar results were obtained with mouse fibroblasts (Swiss 3T3 cells).

The present study demonstrates that treatment of cells with cAMP-elevating agents can lead to a substantial change in the level of the LUC activity independently of a transcriptional activation of promoter elements. The results presented here suggest that variations in LUC activity in studies involving cAMP-elevating agents should be interpreted with caution. Hence we propose that, if this reporter-gene system is to be used in such studies, additional controls, including another reporter-gene system under the control of the same promoter elements, should be included. In light of the data presented here and using a reporter gene other than the LUC system, it would be important to re-examine the conclusions of many reports describing the transcriptional activation of various promoter elements by cAMP-elevating agents.

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