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Modulation by cellular cholesterol of gene transcription via the cyclic AMP response element

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Abstract

The effect of rapid changes in cellular cholesterol content on adenosine 3′,5′-cyclic monophosphate (cAMP) response element-mediated gene transcription was investigated. The study was carried out in Chinese hamster ovary (CHO-K1) cells permanently expressing the human β_2-adrenoceptor. Gene transcription was quantified using a reporter gene (secreted placental alkaline phosphatase) under the transcriptional control of cAMP response element (CRE) sequences. Cellular cholesterol was reduced by 42% or elevated by 47% by incubating cells for 1 hr with methyl-β-cyclodextrin alone or methyl-β-cyclodextrin complexed with cholesterol, respectively. There was a significant negative correlation between the free cholesterol content of the cells and CRE-mediated gene expression in response to 10^{-6} M isoprenaline (slope = -4.57 ± 0.73, $P < 0.001$), indicating that β_2-adrenoceptor-mediated activation of the CRE is inhibited by cholesterol. Cyclic AMP accumulation in response to isoprenaline (10^{-12} to 10^{-5} M) was also inhibited in cholesterol-enriched cells and enhanced in cholesterol-depleted cells compared to controls ($P < 0.05$, two-way ANOVA). Cholesterol also inhibited serum-mediated enhancement of CRE-driven gene expression, and we present data suggesting that the pathway activated by serum and inhibited by cholesterol could be independent of adrenoceptor activation and protein kinase A. We conclude that in CHO-K1 cells cholesterol inhibits at least two processes that can stimulate CRE-mediated gene expression. One is isoprenaline activation of cAMP synthesis, the other is activated by serum. These findings demonstrate that activation of gene transcription by extracellular stimuli could be influenced by cellular cholesterol content. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Cyclic AMP response element; CRE; Transcription; Cholesterol; β_2-Adrenoceptor; β-Cyclodextrin; Cyclic AMP

1. Introduction

The activities of a number of plasma membrane receptors and other plasma membrane proteins can be modulated by changes in the cholesterol content of their environment. Such modulation has been shown for the transferrin receptor [1], the nicotinic acetylcholine receptor [2], rhodopsin [3], the oxytocin receptor [4], cholecystokinin receptors [4], and adenylyl cyclase [5]. Cellular cholesterol levels have been shown to regulate the transcription of genes involved in lipid metabolism via the sterol regulatory element (reviewed in [6,7]). However, little is known about the indirect impact of cholesterol on gene transcription via its effects on plasma membrane proteins involved in signalling pathways from the extracellular environment to the nucleus.

CRE-mediated gene transcription is an example of transcriptional response to activation of plasma membrane proteins by extracellular stimuli. CREs are present in the promoters of many genes, including those for c-*fos*, somatostatin, phosphoenolpyruvate carboxykinase, tyrosine hydroxylase, vasoactive intestinal peptide, pro-enkephalin, and α-chorionic gonadotropin [8]. Consequently, CRE-mediated transcription is involved in a variety of cellular responses, including cell proliferation, intermediary metabolism, neuronal signalling, and apoptosis [9]. The transcriptional activation of CREs is regulated via a family of transcription factors, the CRE-binding (CREB) proteins, which become active upon phosphorylation on serine 133.

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; CHO, Chinese hamster ovary; CRE, cyclic AMP response element; FBS, foetal bovine serum; MβCD, methyl-beta-cyclodextrin: PKA, protein kinase A; SPAP, secreted placental alkaline phosphatase.

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PKA was the first activator of CREB to be described. Subsequently, it was found that other kinases can also phosphorylate CREB, thus enhancing CRE-mediated transcription. Serine 133 of CREB can be phosphorylated by Ca^{2+}/calmodulin-dependent kinases [10], protein kinase C [11], RSK2 [12], and p38 MAP (mitogen-activated protein) kinase [13]. The proteins involved in cAMP production are all plasma membrane proteins (receptors, G-proteins, adenylyl cyclase), as are other receptors for growth factors and hormones involved in initiating signalling pathways that activate the protein kinases mentioned above.

Recent studies have provided information relating to the organization of lipids and proteins in the plasma membrane into microdomains and their possible interaction with signalling pathways (reviewed in [14–16]). Proteins can be selectively included or excluded from lipid microdomains, providing a mechanism for co-localization of molecules that act sequentially to activate signalling pathways. Changes in cholesterol content alter the composition and distribution of lipid microdomains and their associated proteins, with subsequent changes in signalling cascades.

The cholesterol content of cells can be altered rapidly using cyclic heptamers of glucose, β-cyclodextrins. Cyclodextrins have a hydrophobic cavity that can incorporate non-polar molecules, forming soluble complexes. β-Cyclodextrins have a high affinity for cholesterol compared to other membrane lipids [17]. Incubation of cultured cells or cell membranes with methyl-β-cyclodextrin results in rapid loss of cellular cholesterol [4,18], while incubation with preformed methyl-β-cyclodextrin–cholesterol complex results in rapid gain of cellular cholesterol [4,19]. This movement takes place without insertion of the cyclodextrin molecule into the membrane or its binding to the cell surface [18].

In this study, we investigated the possibility that changes in cellular cholesterol content can modify signalling from extracellular ligands to the nucleus, with subsequent effects on gene transcription. The investigation was carried out in CHO cells that were stably transfected with a reporter gene under the transcriptional control of six CRE sequences and with the human β_2-adrenoceptor (cell line CHO β_2/4 [20]). The sequence of the CRE-containing fragment did not contain sterol regulatory elements or serum response elements. As far as we are aware, the interaction between cellular cholesterol levels and CRE-mediated gene expression has not been investigated before.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium/nutrient mix F-12 (1:1), FBS, BSA (A-2153), MβCD, cholesterol (C-8667), (±)-isoproterenol (I-5627), *p*-nitrophenyl phosphate (104-0, phosphatase substrate), and ICI 118551, ([±]-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxyl-3-[(1-methylethyl)amino]-2-butanol) HCl, were purchased from Sigma. Rolipram was from RBI. H89, {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulphonamide, 2HCl} was from Calbiochem. CPT-cAMP (8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphate) was from Roche. Cholesterol esterase (cat. no. 393916) and cholesterol assay kits (cat. no. 1 442 341 and 139 050) were from Boehringer Mannheim. [adenine-U-^{14}C]cAMP and [8-^{3}H]adenine were from Amersham International. [^{125}I]Iodocyanopindolol was from NEN Life Science Products Ltd.

2.2. Cells and media

This study used CHO cells expressing the cDNA for the human β_2-adrenoceptor and containing a reporter gene (SPAP) downstream of a minimal thymidine kinase promoter under control of six CRE sequences. We have previously described the generation of this cell line (CHO β_2/4 cells [20]). The cells were routinely cultured in FBS medium (see below) in 75-cm^2 flasks at 37°, in an atmosphere of 5% CO_2/95% air. Experiments were carried out using confluent cells in 24-well cluster dishes, unless stated otherwise.

Three types of media were used in the study: 1) FBS medium: Dulbecco's modified Eagle's medium/nutrient mix F-12 (1:1) containing 10% FBS and 2 mM L-glutamine; 2) BSA medium: Dulbecco's modified Eagle's medium/nutrient mix F-12 (1:1) containing 5 mg/mL of BSA and 2 mM L-glutamine; and 3) unsupplemented medium: Dulbecco's modified Eagle's medium/nutrient mix F-12 (1:1) containing 2 mM L-glutamine.

2.3. Enrichment and depletion of cellular cholesterol

Cholesterol-depleting and -enriching procedures, as well as subsequent incubations with agonists and antagonists, were carried out in medium that did not contain serum. Serum-free media were used to avoid exchange of cholesterol between serum lipoproteins and MβCD, or serum lipoproteins and cells. Such transfers would reduce the effectiveness of the cholesterol-loading/unloading procedures.

We obtained efficient exchange of cholesterol between cells and MβCD in serum-free medium containing 5 mg/mL of BSA (Table 1). Although BSA has some capacity to associate with cholesterol, this did not interfere with the cholesterol-depleting and -enriching procedures. We also found that SPAP production in response to isoprenaline in medium containing 10% FBS was very similar to the response in medium containing 5 mg/mL of BSA (data not shown). Therefore, unless stated otherwise, experimental procedures were carried out in serum-free medium that contained 5 mg/mL of BSA (BSA medium).

Cells were seeded into 24-well cluster dishes and cultured in medium containing 10% FBS until confluent. The

confluent cells were incubated with 1 mL BSA medium per well at 37° for 20 min to allow dissociation or internalization of serum lipoproteins. The cultures were then incubated for 1 hr with 1 mL BSA medium alone (control), 1 mL BSA medium containing 2 mM MβCD (cholesterol-depleting medium), or 1 mL MβCD–cholesterol complex solution (cholesterol-enriching medium; prepared as described below). The control, cholesterol-depleted, and cholesterol-enriched cells were then washed with 1 mL BSA medium to remove the MβCD and MβCD–cholesterol complex before any subsequent experimental procedures were carried out.

Preparation of MβCD–cholesterol complex was carried out essentially as described in [19]. To prepare 10 mL of complex solution, 48 μL of 50 mg/mL of cholesterol in chloroform:methanol 1:1 was placed in a sterile glass tube and dried in a thin layer on the tube wall, with a gentle stream of N_2. Ten milliliters of 5 mM MβCD in BSA medium, prewarmed to 37°, was added and the tube sonicated in a bath sonicator for 5–10 min. The mixture was then incubated at 37° overnight with mixing, followed by filtration (0.22-μm filter) to remove undissolved cholesterol. The cholesterol content of the filtered medium was assayed with Boehringer Mannheim cholesterol assay kit cat. no. 1 442 341. This procedure yielded 130–160 μg/mL of cholesterol. The starting molar ratio of MβCD to cholesterol was 8:1. The molar ratio in the final filtered solution was 12:1–15:1. The complex was prepared anew for each experiment.

2.4. Measurement of SPAP

Following manipulation of cellular cholesterol as described above, cells were incubated with 1 mL fresh medium, with or without agonists as specified in the legends to tables and figures. Unless stated otherwise, the incubation continued for 6 hr, at which point the medium was removed and stored at −20° for subsequent SPAP assay. When ICI 118551 or H89 was used, cholesterol-depleted, cholesterol-enriched, and control cells were preincubated for 30 min with or without these compounds, prior to incubation for 6 hr with fresh medium with or without ICI 118551, H89, or agonist as appropriate. Note that neither cholesterol nor MβCD was present in the medium during the incubation with agonists.

SPAP activity was assayed as described previously [20, 21]. Medium samples were incubated at 65° for 30 min to inactivate endogenous alkaline phosphatase activity, which is present in serum and commercial preparations of BSA. SPAP activity was then assayed in 20 μL of medium, incubated for 2 hr with the substrate *p*-nitrophenol phosphate. Results are expressed either as percent of basal SPAP secretion in control cells in the same experiments or as mU/well [20,21].

In some experiments, after the removal of medium, the cells were washed with 1 mL PBS and stored at −20° for subsequent assay of cholesterol, protein, or residual SPAP activity in cells. To assay SPAP activity in cells, washed monolayers were lysed with 200 μL of Promega Reporter Lysis Buffer (E397A) for 1 hr. SPAP activity was assayed in 20 μL lysate.

2.5. Cholesterol and protein assays

In some experiments, both SPAP secretion and the cholesterol content of cells were assessed. Immediately after the cholesterol-loading/unloading procedure (t = 0) or following the subsequent 6-hr incubation with or without agonists (t = 6), the medium was removed, and the cells washed with 1 mL PBS and stored at −20°. Lipids were extracted from the cell monolayers with 2 × 1 mL isopropanol. Extracts from each well were pooled, dried down, and stored at −20°. The free cholesterol content of the extracts was assayed using a Boehringer Mannheim free cholesterol colourimetric assay kit (cat. no. 139 050). The kit protocol was scaled down for use in 96-well plates. Total cholesterol content was assayed following the addition of cholesterol esterase to the system to hydrolyze cholesterol esters. Dried cellular lipid extracts were dissolved in 50 μL isopropanol and 0.6 mL of kit reagent added. The reagent–sample mixture (0.3 mL) was placed in each of 2 adjacent wells of a 96-well plate. Cholesterol oxidase (2.5 μL, supplied with the kit) was added to one of the wells, with the other well acting as sample blank. Optical density at 405 nm was read, following incubation for 1 hr at 37°. This represents the free cholesterol in the sample. Cholesterol esters were then hydrolyzed by adding 1 μL of 2 mg/mL of cholesterol esterase. The optical density at 405 nm was read following further incubation for 1 hr at 37°, giving an estimate of the total cholesterol (free + esterified) content of the sample. Standard solutions of known cholesterol concentration were included with each assay. The validity of this procedure was confirmed by assaying mixtures of cholesterol and cholesterol ester of known composition.

Following lipid extraction, the cells were left to dry at room temperature to remove any remaining isopropanol. Cellular proteins were then dissolved in 1 mL 0.1 M NaOH and assayed using the method of Lowry *et al*. [22] with BSA as standard.

2.6. Cyclic AMP accumulation

Elevation of cAMP was assessed by measurement of the amount of [^{3}H]adenine converted to [^{3}H]cAMP [23]. Cells were incubated with 1 mL BSA medium containing 2 μCi/mL of [^{3}H]adenine for 1 hr, followed by 1 hr with 1 mL cholesterol-loading, -unloading, or control medium containing 2 μCi/mL of [^{3}H]adenine. The wells were then washed with 1 mL Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4 (HBH). The washed cells were incubated with 1 mL HBH containing 10 μM rolipram for 30 min to inhibit phosphodiesterase activity. Isoprenaline was added in 10 μL to the wells and the incubation continued for a

further 10 min. The reaction was stopped by the addition of 40 μL 70% $HClO_4$. [^{3}H]cAMP was isolated by sequential chromatography on Dowex and alumina columns [24] using 2000 dpm [^{14}C]cAMP as a recovery standard. In experiments measuring basal activity with and without ICI 118551, the concentration of labelling [^{3}H]adenine was increased to 3 μCi/mL. ICI 118551 was added at the same time as rolipram.

2.7. [^{125}I]Iodocyanopindolol binding

Confluent cells in 75-cm^2 flasks were enriched or depleted of cholesterol following the procedure described above for 24-well cluster dishes, with 15 mL of the appropriate medium used per flask. Specific radioligand binding was assayed in membrane preparations from these cells, with non-specific binding determined in the presence of 1 μM propranolol [20].

2.8. Data analysis

Statistical significance was analysed using unpaired *t*-test, paired *t*-test, or analysis of variance (ANOVA) as stated in the legends to figures and tables. Assessment of linear regression, correlation, and fitting of agonist concentration–response curves to four parameter logistic equations were carried out using Prism 2 (GraphPad Software).

3. Results

3.1. Influence of cellular cholesterol content on isoprenaline-induced gene expression

Free cholesterol concentrations of CHO β_2/4 cells were manipulated as described in Materials and Method to achieve a range of 15–40 μg cholesterol per mg cell protein. The amount of the reporter gene product, SPAP, was reduced with increasing cellular free cholesterol. Fig. 1 demonstrates a linear negative correlation between the free cholesterol content of the cells and the amount of SPAP secreted into the medium in response to 10^{-6} M isoprenaline (closed symbols, slope -4.57 ± 0.73, $r^2 = 0.64$, $P < 0.001$). This concentration of isoprenaline was chosen to give maximal activation of the receptor. In the absence of agonist, there was a small effect of free cholesterol on SPAP secretion as shown by the shallow (though significantly different from zero) slope of the regression line (open symbols, slope -0.93 ± 0.22, $r^2 = 0.35$, $P < 0.001$). The data indicated that the production of SPAP in response to isoprenaline was attenuated by cholesterol. Using the parameters derived from the regression lines, it can be calculated that 10^{-6} M isoprenaline stimulated SPAP production by 2.0-fold over basal (i.e. 100% above basal) in cells containing 20 μg cholesterol per mg protein, while at 40 μg/mg the stimulation was only 1.35-fold (i.e. 35% above basal). Thus, the difference in cholesterol content resulted in 75% reduction in the effectiveness of the response to isoprenaline.

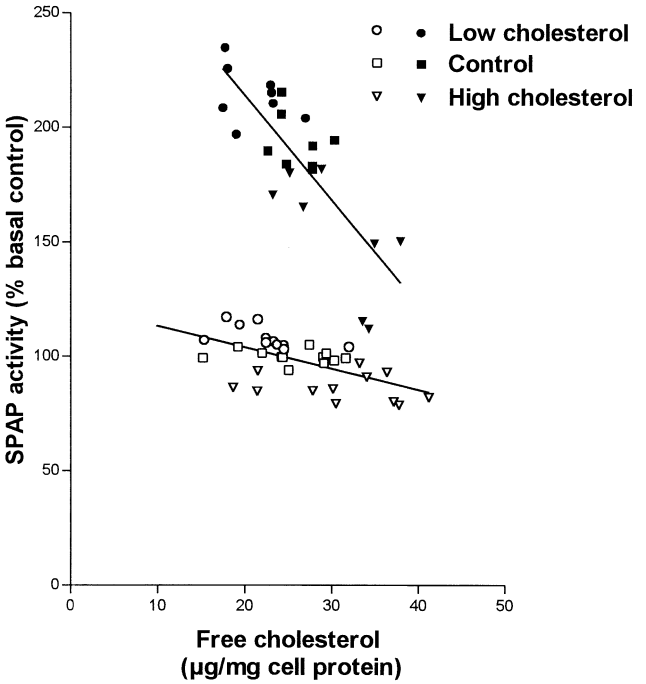

Fig. 1. Isoprenaline-stimulated and basal SPAP secretion as a function of cellular free cholesterol. Cells were depleted or enriched with cholesterol over 1 hr, then washed and incubated in fresh BSA medium for 6 hr with (closed symbols) or without (open symbols) 10^{-6} M isoprenaline. At the end of the incubation, SPAP activity was assayed in the medium. Cellular lipids were extracted and the free cholesterol content of the cells determined. All procedures were as described in Materials and Methods. Each point is derived from 1 well in which both SPAP activity and cellular free cholesterol were assayed. Data are from 4 independent experiments. SPAP activity is expressed as % of basal activity measured in triplicate control cultures in the same experiment. The range of basal activities was 0.0070–0.0116 mU/well.

3.2. Changes in cholesterol content of CHO β_2/4 cells following treatment with MβCD

Table 1 shows the changes in cellular cholesterol associated with the procedures followed in the experiments presented in Fig. 1. Incubation of CHO β_2/4 cells for 1 hr with 2 mM MβCD resulted in cholesterol depletion, and incubation for 1 hr with 5 mM MβCD–cholesterol complex resulted in cholesterol enrichment. In both cases, statistically significant changes of 30–40% compared to controls were observed in free and total cholesterol (Table 1, t = 0).

Following removal of the cholesterol-depleting or -enriching medium and subsequent 6-hr incubation in control BSA medium alone, the cholesterol-depleted cells gained free cholesterol and the cholesterol-enriched cells lost free cholesterol (Table 1, free cholesterol at t = 6 hr compared to t = 0). The difference in free cholesterol content between treated and control cells at t = 6 hr was reduced to 16–17%, but remained statistically significant (Table 1).

Table 1
Cholesterol content of CHO β_2/4 after treatment with MβCD

Treatment of cells	Free cholesterol (μg cholesterol/well)		Total cholesterol (μg cholesterol/well)	
	t = 0 hr (N = 6)	t = 6 hr (N = 7)	t = 0 hr (N = 4)	t = 6 hr (N = 6)
MβCD (Low cholesterol cells)	2.2 ± 0.1***	3.0 ± 0.2*·*****	2.6 ± 0.1**	3.2 ± 0.3**·*****
No additions (Control cells)	3.8 ± 0.2	3.6 ± 0.2	4.3 ± 0.1	4.2 ± 0.2
MβCD:cholesterol complex (high cholesterol cells)	5.6 ± 0.5**	4.2 ± 0.2*·****	5.7 ± 0.5*	5.0 ± 0.2**

Cellular lipids were extracted from cells either immediately after the 1-hr cholesterol depletion/enrichment procedure (t = 0) or following this procedure and a subsequent 6-hr incubation in BSA medium alone. Free and total cholesterol were assayed in the extracts (total cholesterol = free cholesterol + esterified cholesterol). All procedures were as described in Materials and Methods. Values are means ± SEM from N independent experiments in each of which 2–4 wells were assigned to each experimental condition.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control values in the same column; unpaired t-test.

**** $P < 0.05$, ***** $P < 0.01$ compared to values at t = 0 in the same row; unpaired t-test.

The use of medium without serum lipoproteins minimizes transfer of cholesterol in or out of the cells. Consequently, the changes observed during the 6-hr incubation following the depletion/enrichment procedures must have been due primarily to endogenous processes. These changes were compatible with cholesterol-depleted cells synthesizing cholesterol and cholesterol-enriched cells esterifying cholesterol (Table 1). It is relevant that in the cholesterol-enriched cells, the main difference was due to conversion of free cholesterol to cholesterol ester, with very little actual loss of cholesterol from cells. We did not add inhibitors of cholesterol synthesis or cholesterol esterification, since significant changes in free cholesterol content and subsequent responses to isoprenaline were observed in the absence of such inhibitors.

3.3. Interaction between cellular cholesterol and secretion of SPAP from CHO β_2/4 cells into the medium

SPAP is much used as a reporter gene to study transcriptional control [21]. It is secreted into the medium, allowing sampling without lysis of the cells. Cholesterol is a factor in determining the physical properties of membranes and therefore could affect the secretion process directly. Consequently, cholesterol-induced differences in SPAP accumulation in the medium could reflect changes in the rate of secretion, as well as changes in gene expression.

We assessed the relationship between SPAP secretion into the medium and the residual cellular SPAP of cholesterol-enriched, control, and cholesterol-depleted cells. 96.8% ± 0.56 (mean ± SD, N = 48) of total (cells + medium) SPAP activity in the well was found in the medium. The amount of SPAP in the medium increased linearly with increasing cellular SPAP concentration regardless of the cholesterol status of the cells (Fig. 2), indicating that the secretion process is not impaired or stimulated by changes in cellular cholesterol. Thus, the activity of SPAP in the medium accurately reflects CRE-driven transcriptional control of the reporter gene over the range of cholesterol concentrations used in this study.

3.4. Effects of cellular cholesterol content on cAMP accumulation and SPAP production following exposure to 10^{-12}–10^{-5} M isoprenaline

The dose–response curves of cAMP accumulation over a range of 10^{-12}–10^{-5} M isoprenaline showed stimulation in cholesterol-depleted cells ($P < 0.01$) and inhibition in cholesterol-enriched cells ($P < 0.05$) compared to controls (Fig. 3). Maximal cAMP synthesis in response to isoprenaline was inhibited by 27% in cholesterol-enriched cells

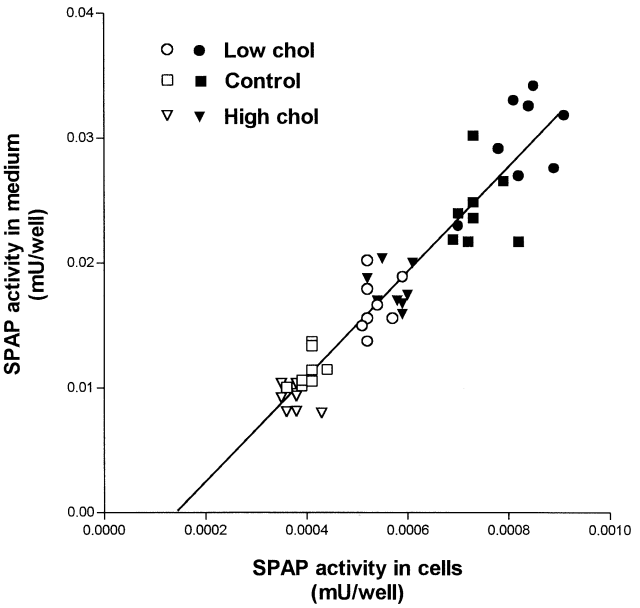

Fig. 2. Correlation between residual SPAP activity in cells and SPAP secreted into the medium. Cholesterol-depleted, cholesterol-enriched, or control cells were incubated for 1 hr with (closed symbols) or without (open symbols) 10^{-6} M isoprenaline. The cells were washed for 1 hr with 1 mL BSA medium and the incubation continued in 1 mL fresh BSA medium for a further 22 hr. At the end of the incubation, SPAP activity was assayed in the medium and in the cells as described in Materials and Methods. Each point represents one well in which SPAP activity was assayed in both cells and medium. Data are all from one experiment. r^2 = 0.897, $P < 0.001$.

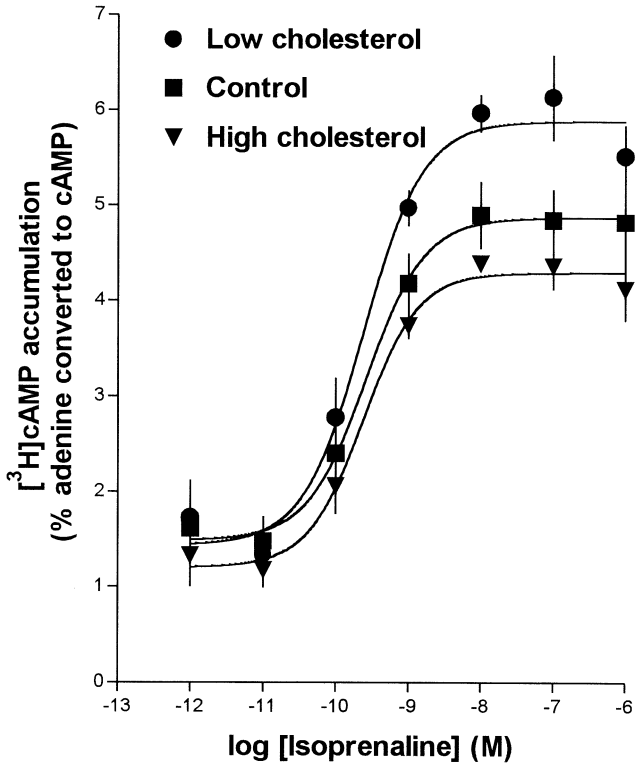

Fig. 3. [^{3}H]cAMP accumulation in response to isoprenaline in cells of different cholesterol content. Cells were labelled with [^{3}H]adenine and their cholesterol content manipulated. [^{3}H]cAMP accumulation was determined following exposure to the indicated concentrations of isoprenaline for 10 min. All procedures were as described in Methods. Values are means ± SEM from 3 independent experiments. Statistical analysis by two-way ANOVA: low cholesterol vs control, $P < 0.01$; high cholesterol vs control, $P < 0.05$.

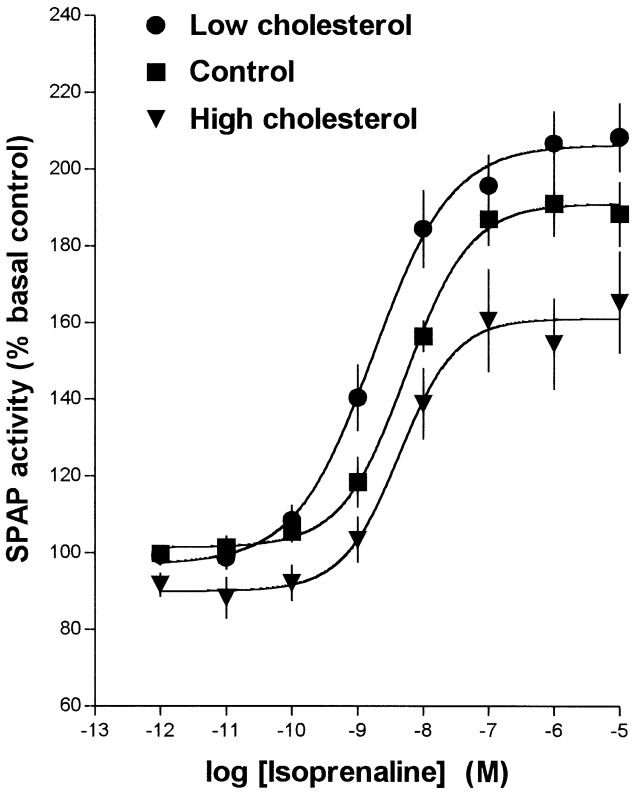

Fig. 4. SPAP activity in response to isoprenaline in cells of different cholesterol content. Cholesterol-depleted, cholesterol-enriched, or control cells were washed and then incubated for 6 hr in BSA medium with the indicated concentrations of isoprenaline. At the end of the incubation, SPAP activity was assayed in the medium. All procedures were as described in Materials and Methods. SPAP activity is expressed as % of basal activity measured in 2–3 control cultures in the same experiment. The range of basal activities was 0.0040–0.0114 mU/well. Values are means ± SEM from 7 independent experiments. Statistical analysis by two-way ANOVA: low cholesterol vs control, $P < 0.001$; high cholesterol vs control, $P < 0.001$.

compared to cholesterol-depleted cells, with no changes observed in EC_{50} (Table 2).

Cholesterol depletion increased SPAP secretion and cholesterol enrichment reduced SPAP secretion over 10^{-12}–10^{-5} M isoprenaline (Fig. 4). High cholesterol significantly reduced the maximal response to isoprenaline without affecting EC_{50}, while low cholesterol significantly increased both the maximal response and the sensitivity to isoprenaline (Table 2). Although the differences observed were small, they were statistically significant. The data in Figs. 3

Table 2
Dose–response parameters for isoprenaline-stimulated cAMP accumulation and SPAP production in cells of different cholesterol content

Parameter	Low cholesterol cells	Control cells	High cholesterol cells
Cyclic AMP accumulation			
Minimum (% conversion)	1.53 ± 0.61	1.62 ± 0.23	1.24 ± 0.42
Maximum (% conversion)	5.93 ± 0.46	4.95 ± 0.54	4.35 ± 0.29***
−Log EC_{50}	9.84 ± 0.22	9.97 ± 0.52	9.88 ± 0.36
SPAP production			
Minimum (% of basal control)	101.4 ± 3.8	98.7 ± 2.2	88.8 ± 4.9*
Maximum (% of basal control)	205 ± 9.2**	190.2 ± 9.8	162.1 ± 13.7*,***
−Log EC_{50}	8.75 ± 0.23*	8.37 ± 0.16	8.46 ± 0.21

Values are means ± SEM of parameters, derived as described in Materials and Methods, for each of the dose–response experiments presented in Fig. 3 (cAMP accumulation, N = 3) and Fig. 4 (SPAP production, N = 7). Statistical significance was assessed by paired *t*-test.

* $P < 0.05$, ** $P < 0.01$ with respect to control cells.

*** $P < 0.01$ with respect to cholesterol-depleted cells.

Table 3
Basal cAMP and SPAP production in cells of different cholesterol content ± ICI 118551

		N	Low cholesterol cells	Control cells	High cholesterol cells
Cyclic AMP production	Basal	3	110.4 ± 9.5	100	94.5 ± 8.4
	Basal + ICI 118551	3	33.1 ± 14.1**	27.3 ± 4.2	35.1 ± 6.9*
SPAP production	Basal	9	104.5 ± 2.1	100	87.3 ± 2.5***
	Basal + ICI 118551	9	91.4 ± 2.2**	88 ± 2.1	81.9 ± 2.2**,***

Cyclic AMP and SPAP production were measured in cholesterol-depleted and cholesterol-enriched cells in the presence or absence of 10^{-6} M ICI 118551. All procedures were as described in Materials and Methods. Values are means ± SEM from N independent experiments and are expressed as percentages of basal control values in the same experiment. In each experiment, 3–4 wells were assigned to each experimental condition. The range of basal cAMP accumulation in control cultures was 0.51–1.07% of [^{3}H]adenine converted to [^{3}H]cAMP in 40 min. The range of basal SPAP production in control cultures was 0.004–0.012 mU/well in 6 hr.

* $P < 0.05$ and ** $P < 0.01$ compared to basal activity in the same column. *** $P < 0.01$ compared to low cholesterol cells in the same row. Statistical significance was assessed by paired *t*-test.

and 4 suggest that cholesterol modulation of SPAP production could be consequent to its effects on cAMP levels.

The characteristics of the specific binding of [^{125}I]iodocyanopindolol to membranes prepared from cholesterol-depleted, control, or cholesterol-enriched cells were very similar (B_{max} = 959 ± 137, 792 ± 61, 723 ± 80 fmol/mg and K_d = 113 ± 34, 77 ± 15, 60 ± 18 pmol/L for cholesterol-depleted, control, and cholesterol-enriched cells, respectively [mean ± SEM, N = 5]). Thus, the expression of the β_2-adrenoceptor was not altered following 1 hr of cholesterol-loading/unloading treatment.

3.5. Effects of cellular cholesterol content on cAMP accumulation and SPAP production in the absence of β_2-adrenoceptor agonist

Agonist-independent activity of the β_2-adrenoceptor in CHO β_2/4 cells is significant and can be inhibited with ICI 118551, a β_2-adrenoceptor antagonist with inverse agonist activity [20]. We investigated the possibility that cholesterol can alter this constitutive activity. ICI 118551 inhibited basal cAMP accumulation down to 27–35% of control values in all cells, regardless of their cholesterol content (Table 3). Basal cAMP production was not significantly altered by cholesterol, either in the presence or absence of ICI 118551. Thus, cholesterol does not play a significant role in determining the level of β_2-adrenoceptor constitutive activity (assessed by cAMP accumulation).

The substantial inhibition of basal cAMP accumulation by ICI 118851 was associated with a rather smaller inhibition of basal SPAP secretion. Whereas ICI 118851 inhibited cAMP levels down to 27–35% of controls, SPAP production was inhibited only down to 80–90% of control values. The differences in basal SPAP secretion between cholesterol-enriched and cholesterol-depleted cells were small but statistically significant, both in the presence and absence of ICI 118851 (Table 3).

The data in Table 3 suggest that in the absence of β_2-adrenoceptor agonist, CRE-mediated gene expression in CHO β_2/4 cells is not very sensitive to changes in cAMP. Unlike isoprenaline-stimulated SPAP production, which follows a similar pattern to isoprenaline-stimulated cAMP accumulation (Fig. 4 compared to Fig. 3), the changes in basal cAMP accumulation induced by ICI 118551 were not associated with parallel changes in basal SPAP production (Table 3).

3.6. Interaction between cellular cholesterol content and stimulation of SPAP production by CPT-cAMP

We have demonstrated that cholesterol modulates isoprenaline-stimulated cAMP accumulation (Fig. 3), with broadly similar effects on isoprenaline-stimulated SPAP production (Fig. 4). CPT-cAMP, a membrane-permeant cAMP analogue, directly activates PKA, by-passing β-adrenoceptor activation of endogenous cAMP synthesis. If the effects of cholesterol on SPAP production are wholly consequent to its effects on cAMP levels, then cholesterol should not alter CPT-cAMP-mediated SPAP production.

However, the effects of changes in cellular cholesterol content on CPT-cAMP-stimulated SPAP production (Fig. 5A) were similar to those observed on SPAP production in response to isoprenaline (Fig. 4). Maximal SPAP production in response to CPT-cAMP was increased in cholesterol-depleted cells and reduced in cholesterol-enriched cells (Table 4). There was a negative correlation between cellular free cholesterol and CPT-cAMP-stimulated SPAP production, comparable to the negative correlation between free cholesterol and isoprenaline-induced SPAP production in the same experiments. (CPT-cAMP [2.7 mM]: slope −5.28 ± 0.81, r^2 = 0.86, $P < 0.01$; isoprenaline [10^{-6} M]: slope −5.41 ± 1.06, r^2 = 0.79, $P < 0.001$; basal: slope −2.10 ± 0.44, r^2 = 0.77, $P < 0.01$, experimental data not shown).

In order to minimize the contribution of constitutive β-adrenoceptor activity, SPAP production was also assessed in cultures exposed to CPT-cAMP (3.7×10^{-6}–2.7×10^{-3} M) with the addition of ICI 118851 (10^{-6} M). ICI 118851 reduced CPT-cAMP-mediated SPAP secretion in all cultures, but the differences between cholesterol-

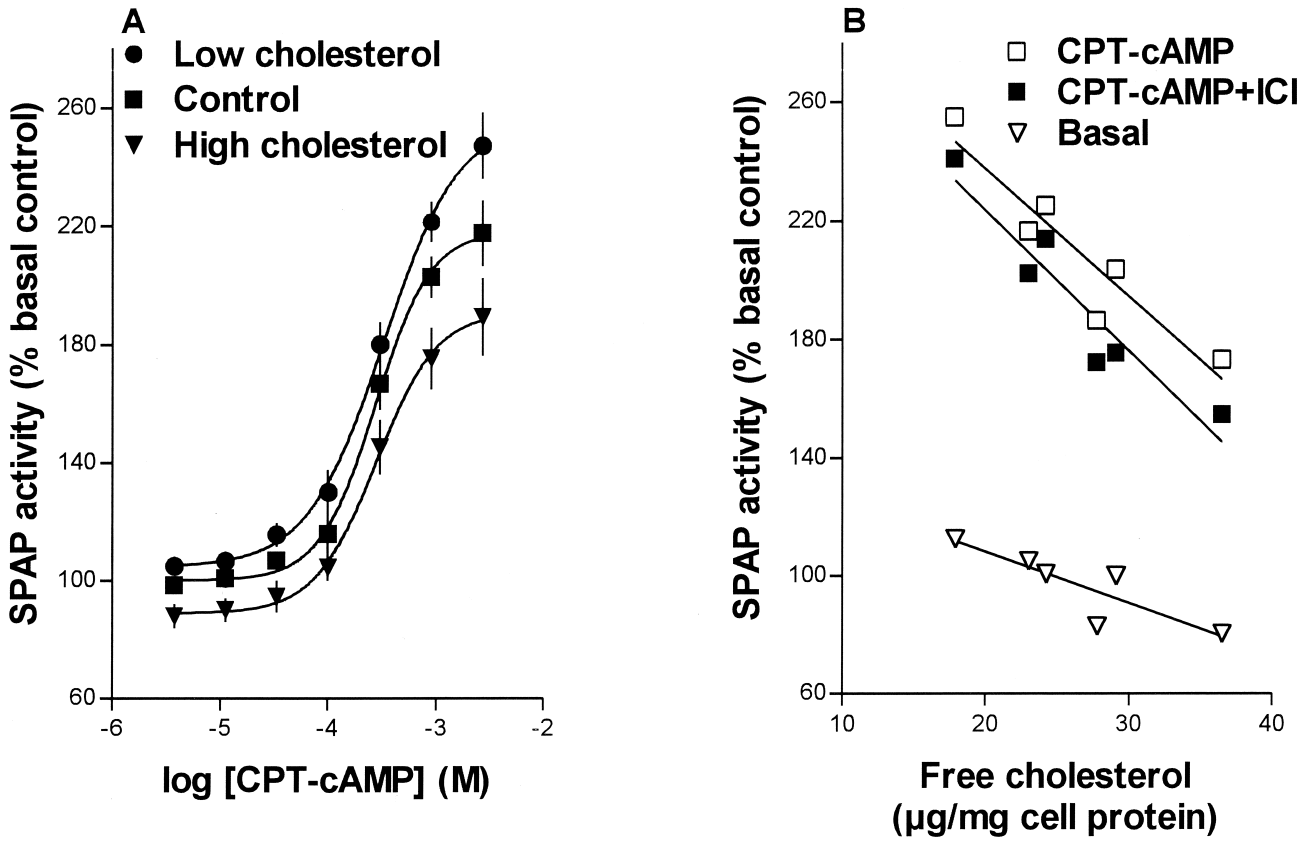

Fig. 5. SPAP production in response to CPT-cAMP. (A) Cholesterol-depleted, cholesterol-enriched, or control cells were washed and then incubated for 6 hr in BSA medium containing the indicated concentrations of CPT-cAMP. At the end of the incubation, SPAP activity was assayed in the medium. All procedures were as described in Materials and Methods. SPAP activity is expressed as % of basal activity measured in triplicate control cultures in the same experiment. The range of basal activities was 0.0036–0.0121 mU/well. Values are means ± SEM from 6 independent experiments. Statistical analysis by two-way ANOVA: low cholesterol vs control, $P < 0.01$; high cholesterol vs control, $P < 0.01$. (B) Cholesterol-depleted, cholesterol-enriched, or control cells were washed and then incubated for 6 hr in BSA medium with 2.7 mM CPT-cAMP, 2.7 mM CPT-cAMP and 10^{-6} M ICI 118551, or with no additions. At the end of the incubation, SPAP activity was assayed in the medium. Data are from 2 independent experiments. The cholesterol content of cholesterol-depleted, cholesterol-enriched, and control cells was determined in duplicate wells in each experiment. SPAP activities are plotted against the mean cholesterol content of the appropriate group in the same experiment. All procedures were as described in Materials and Methods.

enriched, cholesterol-depleted, and control cells were maintained (data not shown). The negative correlation between cellular free cholesterol and CPT-cAMP-stimulated SPAP production was maintained in the presence of 10^{-6} M ICI 118851 (Fig. 5B, CPT-cAMP: slope −4.31 ± 0.83, $r^2 = 0.87$, $P < 0.01$; CPT-cAMP + ICI 118851: slope −4.75 ± 0.82, $r^2 = 0.89$, $P < 0.01$; basal: slope −1.74 ± 0.50, $r^2 = 0.75$, $P < 0.05$). The data in Fig. 5 indicate that a β_2-adrenoceptor-independent pathway that activates CRE-mediated gene expression is also influenced by cellular cholesterol content.

3.7. Interaction between cellular cholesterol content and stimulation of SPAP production by serum components

We have previously shown that CHO β_2/4 cells with access to serum produce more SPAP than serum-deprived

Table 4
Dose–response parameters for CPT-cAMP-stimulated SPAP production in cells of different cholesterol content

Parameter	Low cholesterol cells	Control cells	High cholesterol cells
Minimum (% of basal control)	102 ± 4.5	98.7 ± 2.8	88.3 ± 4.7
Maximum (% of basal control)	252.6 ± 17.8*	219 ± 13.4	191.5 ± 13.8**·***
$-\text{Log}\ EC_{50}$	3.51 ± 0.08	3.57 ± 0.07	3.56 ± 0.10

Values are means ± SEM of parameters, derived as described in Materials and Methods, for each of the 6 dose–response experiments presented in Fig. 5A. Statistical significance was assessed by paired *t*-test.

* $P < 0.05$, ** $P < 0.01$ with respect to control cells. *** $P < 0.05$ with respect to cholesterol-depleted cells.

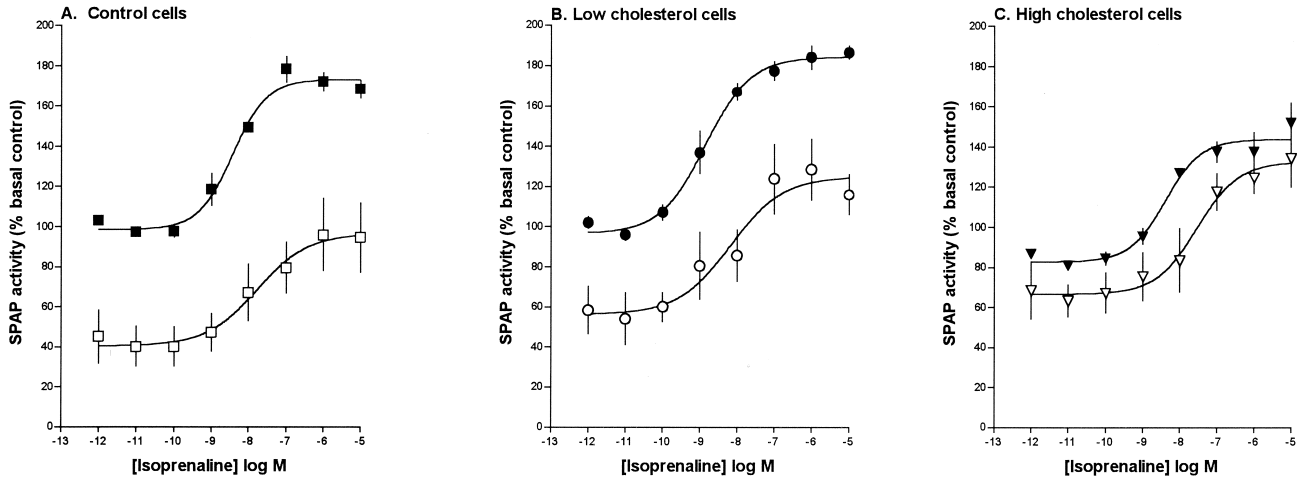

Fig. 6. Effect of serum on isoprenaline-stimulated SPAP production in cells of different cholesterol content. SPAP production during 6-hr incubation with 10^{-12}–10^{-5} M isoprenaline was assessed in cells subjected to our standard procedure (closed symbols; cultured in FBS medium, followed by experimental procedures in BSA medium) or cells deprived of serum and BSA (open symbols; 24 hr in unsupplemented medium, followed by experimental procedures in unsupplemented medium). SPAP activity is expressed as % of basal control activity in BSA medium measured in 2–3 control cultures in the same experiment. The range of basal activities was 0.0040−0.0114 mU/well. Values are means ± SEM from 4 independent experiments. Statistical analysis by two-way ANOVA comparing serum-deprived cells (open symbols) versus cells with access to serum/BSA (closed symbols): panel A, $P < 0.001$; panel B, $P < 0.001$; and panel C, $P < 0.001$.

cells, both in the presence and absence of isoprenaline [20]. Fig. 6 shows that cholesterol enrichment of cells inhibited serum-mediated enhancement of SPAP production. Serum enhancement of SPAP production in cholesterol-depleted cells (Fig. 6B) was broadly similar to that seen in control cells (Fig. 6A). However, in cholesterol-enriched cells, these effects were substantially attenuated (Fig. 6C).

Table 5 demonstrates that the process by which serum enhances the reporter gene expression is not mediated via PKA, since it is not inhibited by H89. Stimulation of SPAP production by isoprenaline was very effectively inhibited by H89, both in serum-deprived and control cells. However, the difference in SPAP production between serum-deprived cells and controls (which had access to serum/BSA) was maintained in the presence of H89. The observation that in the absence of isoprenaline SPAP production in control cells was not inhibited by H89 (Table 5) is compatible with the data in Table 3, where changes in basal cAMP accumulation, due to inhibition of constitutive activity, were not associated with similar changes in basal SPAP secretion.

Taken together, the data presented in Figs. 3, 4, and 6 and Table 5 are compatible with cholesterol influencing at least two different processes involved in CRE-mediated gene transcription. The first is cAMP synthesis (Fig. 3), while the second process is activated by serum (Fig. 6) and appears to be independent of PKA (Table 5).

4. Discussion

Sterols, particularly cholesterol, regulate the transcription of genes involved in lipid synthesis and cellular cholesterol acquisition by controlling production of the mature form of the sterol regulatory element binding proteins [6,7]. These proteins enhance transcription by binding to the sterol regulatory element in the promoter region of the relevant

Table 5
Effect of H89 on serum-mediated activation of SPAP production

	SPAP production (μU/well)			
	No H89		20 μM H89	
Isoprenaline	−	+	−	+
Serum-deprived	2.7 ± 0.1*	6.3 ± 0.4*,****	1.0 ± 0.3**	1.3 ± 0.3**
Control	5.2 ± 0.3	11.3 ± 1.3***	6.7 ± 0.7	7.4 ± 0.8

Control cells were cultured in FBS medium, followed by 30 min in fresh BSA medium ± 20 μM H89. The medium was replaced with 1 mL BSA medium with or without 20 μM H89 or 10^{-6} M isoprenaline and the incubation continued for 6 hr. Serum-deprived cells were cultured for 24 hr in unsupplemented medium and subsequent incubations were as for control cells, but carried out in unsupplemented medium. Values are means ± SEM from 3 independent experiments in each of which 3 wells were assigned to each experimental condition. Statistical significance was assessed by unpaired *t*-test.

* $P < 0.05$, ** $P < 0.01$ with respect to control cells in the same column.

*** $P < 0.05$, **** $P < 0.01$ with respect to cells without isoprenaline.

genes. All of these genes are involved in production or acquisition of lipids (cholesterol, saturated, and mono-unsaturated fatty acids) [6,7]. This study demonstrates that rapid changes in cellular cholesterol content can also play a role in regulating expression of CRE-mediated gene expression.

The experiments were carried out with CHO β_2/4 cells expressing the human β_2-adrenoceptor and containing a reporter gene under the transcriptional control of six CRE elements [20]. We have established by searches against the TRANSFAC database [25] using MatInspector V2.2 [26] and PatSearch 1.1 [27] that the sequence of the CRE-containing fragment [20] did not contain other response elements, in particular sterol regulatory elements [28] and serum response elements [29]. Thus, the observations reported here are independent of the well-described, specific transcriptional control exerted by cholesterol, via the sterol regulatory element, on genes involved in lipid metabolism (reviewed in [6,7]).

We have demonstrated that changes in cellular cholesterol did not affect the secretion process per se (Fig. 2), thus validating the use of the secreted reporter gene, *SPAP*, as a measure of CRE-mediated gene transcription in this study.

Incubation of the cells for 1 hr with 2 mM MβCD or 5 mM MβCD: cholesterol complexes resulted in significant cholesterol depletion and enrichment, respectively (Table 1). Using similar procedures, comparable changes in cellular cholesterol content were observed by others, who demonstrated that the integrity of cultured cells was not compromised following 2-hr exposure to 2 mM MβCD [18] or loss of 70% of cellular cholesterol over a period of 2 hr [30]. Christian *et al*. [19] showed that cholesterol delivered via MβCD–cholesterol complexes was readily esterified, i.e. was metabolically active. In our experiments, the cells responded to changes in their cholesterol as expected, i.e. by acting to normalize their free cholesterol content (Table 1).

There was a negative correlation between cellular cholesterol content and CRE-mediated gene expression (Fig. 1), i.e. activation of the CRE became less efficient with increasing cellular free cholesterol. Cholesterol-dependent changes in isoprenaline-induced gene expression (Fig. 4) were similar to cholesterol-dependent changes in isoprenaline-induced cAMP accumulation (Fig. 3). Isoprenaline-mediated cAMP synthesis involves the plasma membrane proteins, i.e. the β_2-adrenoceptor, the heterotrimeric Gs-protein, and adenylyl cyclase. The data presented in Figs. 3 and 4 suggest that the function of at least one of these proteins could be modulated by cholesterol, with subsequent changes in cAMP-mediated gene transcription.

Cholesterol depletion results in increased membrane fluidity and cholesterol enrichment results in reduced fluidity. Cholesterol depletion with MβCD has been shown to disrupt plasma membrane compartmentalization, caveolin distribution, and lipid rafts [16,31,32]. Thus, in our experiments, cAMP synthesis is enhanced with increased fluidity and disruption of plasma membrane organization. We do not know whether the effects of cholesterol on cAMP synthesis (Fig. 3) are due to changes in membrane fluidity, compartmentalization, caveolin distribution, or to another interaction of cholesterol with the proteins involved (as reported for the oxytocin [4] and nicotinic acetylcholine [2] receptors). It is of interest that our findings are compatible with observations in turkey erythrocytes, where increasing membrane fluidity with cis-vaccenic acid increased both the specific activity of adenylyl cyclase and its activation by adrenaline [33]. In the same system, manipulation of the cholesterol content of the membranes did not alter the maximal binding or the affinity of the β-adrenoceptor [34].

However, our data indicate that the effects of cholesterol on CRE-mediated gene transcription are only partially due to changes in cAMP synthesis. SPAP production in response to the cAMP analogue CPT-cAMP was elevated in cholesterol-depleted cells and inhibited in cholesterol-enriched cells (Fig. 5A). Negative correlation between cholesterol and SPAP production in response to CPT-cAMP was observed both in the presence or absence of ICI 118851, an inhibitor of β-adrenoceptor constitutive activity (Fig. 5B). These observations suggest that in addition to its effects on cAMP synthesis (Fig. 3), cholesterol interacts with another process involved in CRE-mediated transcription that is independent of adrenoceptor activation. This second site of cholesterol action could involve the PKA pathway downstream of cAMP synthesis. One possibility is that cholesterol influences another pathway, independent of cAMP and PKA, and perhaps activated by serum (see below), which acts additively or synergistically with PKA to stimulate the CRE.

We have previously shown that CRE-mediated gene expression in CHO β_2/4 responds not only to β_2-adrenoceptor agonists, but also to the presence of serum. Both the basal and isoprenaline-induced rates of SPAP production are higher in cells incubated with serum than in serum-deprived cells [20]. The data presented in Figs. 1, 2, 4, and 5 were obtained in cells with access to serum/BSA (see Methods for details of experimental protocol). Therefore, the measured values of CRE-mediated gene expression represent the combined effects of serum and isoprenaline (Figs. 1, 2, and 4) or serum and CPT-cAMP (Fig. 5) on CRE-mediated transcription. We demonstrate here that serum-enhanced SPAP production is independent of PKA (Table 5) and that cholesterol enrichment of cells inhibits serum-mediated pathway(s), which activate SPAP secretion (Fig. 6). We have not yet identified the pathway involved in serum-mediated enhancement of CRE-mediated gene expression. As summarized in the introduction, CREB can be phosphorylated and activated by several protein kinases responding to numerous extracellular signals. Preliminary experiments show that serum-mediated activation of CRE is independent of MAP (mitogen-activated protein) kinase, tyrosine kinase, lysophosphatidic acid, and EGF (data not shown).

We conclude that CRE-mediated gene expression in CHO β_2/4 cells correlates negatively with cellular choles-

terol content (Fig. 1). Cholesterol inhibits CRE-mediated gene expression by inhibiting cAMP generation (Fig. 3). Cholesterol also inhibits another as yet unidentified process involved in CRE-mediated gene expression, which is activated by serum (Fig. 6). This study demonstrates that changes in cellular cholesterol levels could influence transcriptional response to extracellular stimuli.

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