Cyclic AMP stimulates the synthesis and function of the low-density lipoprotein receptor in human vascular smooth-muscle cells and fibroblasts

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1. Cyclic AMP-elevating agents stimulate low-density lipoprotein (LDL) receptor activity in human vascular smooth-muscle cells by increasing the rate of receptor protein synthesis. The stimulation is not secondary to the decrease in the regulatory pool of free cholesterol, since it is unaffected, or even enhanced, by inhibition of cholesterol synthesis and esterification, or inhibition of the conversion of cholesterol into its repressor metabolites. The cyclic AMP-mediated up-regulation of the receptor is maintained at low concentrations of inhibitory sterols, but is eventually over-ridden at high concentrations of these sterols. 2. Cyclic AMP-elevating agents also stimulate the hydrolysis of lysosomal cholesterol esters, thus increasing the cellular cholesterol pool and repressing the expression of the LDL receptor. This cholesterol-mediated repressive effect of cyclic AMP can be prevented by chloroquine, which inhibits lysosomal actions, or by ketoconazole, which inhibits conversion of free cholesterol into its repressor metabolite. Thus the cyclic AMP stimulation of the LDL receptor can be masked by the rapid mobilization of free cholesterol from existing cholesterol esters within cultured cells. 3. We have observed that elevated cyclic AMP concentrations will up-regulate the LDL receptor in cholesterol-depleted human vascular smooth-muscle cells, skin fibroblasts and foetal-lung fibroblasts. We propose that our results are evidence for a cyclic AMP-stimulated, sterol-independent, control of LDL-receptor synthesis which is of widespread occurrence in human cells.

INTRODUCTION

The activity of the low-density lipoprotein (LDL) receptor is controlled primarily by cholesterol availability, as described by Brown & Goldstein [1]. In addition, there is an increasing body of evidence showing that cyclic AMP-mediated hormonal effects could play an important role in regulating the expression and activity of the receptor [2–5]. But confusingly, elevated cyclic AMP concentrations have been reported both to inhibit and to stimulate the activity of the LDL receptor in cultured cells. Permeant cyclic AMP analogues have been shown to inhibit the activity of the LDL receptor in HepG2 cells [6], mononuclear leucocytes [5], lung fibroblasts [7] and vascular smooth-muscle cells [8], whereas stimulation was reported in steroid-synthesizing tissues [2], rat hepatocytes [4], human skin fibroblasts [9] and human vascular smooth-muscle cells [9].

We have previously reported a time- and concentration-dependent rise in the number and activity of LDL receptors in cultured human vascular smooth-muscle cells, after exposure to the adenylate cyclase activator forskolin [9]. This effect was exerted on V_{max} values without changing the apparent affinity for LDL. Furthermore, it was abolished in the presence of cycloheximide or actinomycin D, indicating involvement of protein and RNA synthesis.

These changes in LDL receptor activity could be due to a direct action of cyclic AMP on LDL-receptor protein synthesis, or they could be a consequence of cyclic AMP-mediated alterations in sterol supply. Hajjar [10] has demonstrated that elevated cyclic AMP promotes stimulation of lysosomal and cytoplasmic cholesterol esterases in smooth-muscle cells. Thus the confusing diversity of cyclic AMP-mediated changes in LDL receptor activity might reflect a dual role for cyclic AMP in

controlling the receptor: directly via protein synthesis and indirectly by altering sterol supply.

In the present study we have demonstrated the direct stimulation of synthesis of the LDL receptor protein by cyclic AMP-elevating agents in cholesterol-depleted cells. In addition, we have shown that this effect can be masked by the rapid mobilization of free cholesterol from existing cholesterol esters within cultured cells.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium (DMEM) was purchased from Imperial Laboratories (U.K.); foetal-calf serum was from Biological Industries (U.K.) and Flow Laboratories (U.K.); non-essential amino acids were from Flow Laboratories (U.K.); 8-(4-chlorophenylthio)cyclic AMP (CPT-cAMP) was from Boehringer-Mannheim (U.K.); 1,9-dideoxyforskolin was from Calbiochem; [3 H]oleic acid, [14 C]cholesterol oleate, Na 125 I, [35 S]methionine, 14 C-labelled protein molecular-mass standards and mouse anti-(LDL receptor) monoclonal IgG-C7 were purchased from Amersham International (U.K.); collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). BSA, penicillin, streptomycin, amphotericin B, chloroquine, ketoconazole, 25-hydroxycholesterol, dibutyl cyclic AMP (db-cAMP), forskolin, methionine, phenylmethanesulphonyl fluoride, leupeptin and goat anti-mouse IgG were all obtained from Sigma (U.K.). Mevinolin was kindly given by Dr. A. W. Alberts, of Merck Sharp and Dohme (Rahway, NJ, U.S.A.). compound 58035 was kindly given by Dr. J. G. Heider, of Sandoz (Hanover, NJ, U.S.A.).

Abbreviations used: ACAT, acyl-CoA: cholesterol acyltransferase (EC 2.3.1.26); CPT-cAMP, 8-(4-chlorophenylthio)cyclic AMP; db-cAMP, N^6,O^2 -dibutyl cyclic AMP; DMEM, Dulbecco's minimum essential medium; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.88); LPDM, lipoprotein-deficient medium; LDL, low-density lipoproteins.

Cells

Human vascular smooth-muscle cells were released with collagenase from the veins of newly delivered umbilical cords, as described by Gimbrone & Cotran [11]. Human skin fibroblasts were from individuals less than 6 years old, with normal cholesterol metabolism. They were obtained as controls for another study [12]. MRC5 cells were obtained from PHLS Centre for Applied Microbiology and Research, Porton, Salisbury, Wilts., U.K. All details of the culture of smooth-muscle cells, fibroblasts and MRC5 cells are described in [9].

Lipoproteins

Human LDL ($1.019 < d < 1.063$) and lipoprotein-deficient serum ($d > 1.21$) were prepared by sequential centrifugation from freshly collected blood of healthy volunteers [13]. Both fractions were extensively dialysed against 0.15 M-NaCl containing 0.1 mM-EDTA, pH 7.4, before use. Within 1 day of preparation, LDL was radiolabelled with Na¹²⁵I by the ICI method [13], yielding ¹²⁵I-LDL of initial specific radioactivity 100–200 c.p.m./ng. Newly labelled LDL was used within 3 weeks. More than 98% of the ¹²⁵I-labelled material was precipitated by 10% (w/v) trichloroacetic acid, and less than 5% was extractable into lipid solvents.

Lipoprotein-deficient medium (LPDM) consisted of DMEM containing 100 units of penicillin/ml and 100 µg of streptomycin/ml, and human lipoprotein-deficient serum at a protein concentration of 5 mg/ml.

Solutions

db-cAMP and CPT-cAMP were dissolved directly in LPDM just before use. Stock solutions of other agents were stored at –20 °C as follows: mevinolin was converted into its sodium salt as described by Alberts *et al.* [14], and dissolved in ethanol; cholesterol and 25-hydroxycholesterol were dissolved in ethanol; forskolin, dideoxyforskolin, compound 58035 and ketoconazole were dissolved in dimethyl sulfoxide; and chloroquine was dissolved in sterile water. Less than 5 µl of the appropriate stock solution was added per ml of medium to obtain the required concentration of these compounds. The same volume of solvent was added to control cultures.

[³⁵S]Methionine labelling of human vascular smooth-muscle cells

All cells were first washed with 2 ml of DMEM, then preincubated with 2 ml of LPDM for 24 h in order to induce the LDL receptor. Labelling and/or exposure to cyclic AMP-elevating agents followed this preliminary procedure.

(i) **Synthesis of the LDL receptor.** Near-confluent monolayers of human vascular smooth-muscle cells in duplicate 25 cm² flasks were washed with methionine-free DMEM. The cultures were then incubated for 4–24 h, with 2 ml of DMEM containing 5 µM-methionine and lipoprotein-deficient serum at a final protein concentration of 5 mg/ml, with or without cyclic AMP-elevating agents. In some experiments (e.g. Fig. 1) [³⁵S]methionine was added at zero time and the cells were incubated with [³⁵S]methionine together with cyclic AMP-elevating agents for 24 h. To measure the time course of the cyclic AMP effect, [³⁵S]methionine was added directly to the flasks after 0, 12 or 20 h with forskolin or control medium, and the incubation was stopped 4 h later (Fig. 2a). [³⁵S]Methionine was added to a final concentration of 75–200 µCi/ml, giving a specific radioactivity of 15–40 µCi/nmol.

(ii) **Degradation of the LDL receptor protein.** Duplicate flasks of near-confluent cells were washed with methionine-free

DMEM, as above. The cultures were then incubated with 2 ml of DMEM containing 5 mg of lipoprotein-deficient serum/ml, 5 µM-methionine and 180 µCi of [³⁵S]methionine (final sp. radioactivity 36 µCi/nmol)/ml. The cells were labelled for 24 h in this medium. The monolayers were then briefly washed with Dulbecco's phosphate-buffered saline, before incubation with fresh DMEM containing 5 mg of lipoprotein-deficient serum/ml and 500 µM unlabelled methionine, with or without cyclic AMP-elevating agents. The incubations continued for 0, 4, 16 or 24 h.

The labelling (i) or 'cold' chasing (ii) was stopped by chilling the cells on ice and washing each monolayer with 5 ml of ice-cold 10 mM-Hepes (pH 7.4), containing 150 mM-NaCl and 2 mM-CaCl₂. Cells were then lysed with 0.35 ml (per flask) of Buffer B [10 mM-Hepes, pH 7.4, containing 200 mM-NaCl, 2 mM-CaCl₂, 2.5 mM-MgCl₂, 1 mM unlabelled methionine, 1 mM phenylmethanesulphonyl fluoride, 0.1 mM-leupeptin and 1% (v/v) Triton X-100]. Cells were scraped, duplicates were pooled and extracts spun at 150 000 g for 30 min. The small pellet was discarded and the supernatants were taken for immunoprecipitation after measurement of protein content and radioactivity.

Immunoisolation of the LDL receptor protein

This was carried out at 4 °C, as described by Tolleshaug *et al.* [15]. Equal amounts (200–400 µg) of cell-extract protein were incubated with a preformed immune complex prepared from mouse anti-(LDL receptor) IgG C7 and goat anti-mouse IgG. The complex was prepared as described in [15], but with a protein ratio of goat anti-mouse IgG to mouse IgG C7 of 40. Immune complexes containing 50 µg of IgG-C7 were used to precipitate the LDL receptor from extracts of duplicate flasks. The receptor immune complex was precipitated, washed, harvested and dissolved for PAGE exactly as described in [15].

SDS/PAGE and detection of ³⁵S-labelled LDL receptor protein

Samples were heated to 90 °C for 3 min before electrophoresis on 7%-polyacrylamide slab gels in the presence of SDS [16]. Gels were calibrated with the following ¹⁴C-labelled protein standards: carbonic anhydrase (30 kDa), ovalbumin (46 kDa), BSA (69 kDa), phosphorylase *b* (97.4 kDa) and myosin (200 kDa). After electrophoresis, gels were fixed, soaked in 10% (v/v) glycerol for 2 h, impregnated with 1 M-sodium salicylate in 10% (v/v) glycerol for 30 min, dried and stored in contact with pre-flashed X-ray film at –70 °C for 3–10 days. The mature LDL receptor protein appeared as a major band with a molecular mass of 160 kDa [15]. Quantification was with a Helena scanning densitometer.

Cholesterol esterification

The rate of esterification of cellular cholesterol mainly reflects the changes in size of the pool of cholesterol substrate for acyl-CoA:cholesterol acyltransferase (ACAT) [16a]. In one set of experiments (Table 1), the cells were preincubated in LPDM with or without cyclic AMP-elevating agents, to up-regulate the LDL receptor. The medium was then changed to LPDM plus 50 µg of LDL/ml for 6 h, before measurement of cholesterol esterification at 37 °C for 30 min. Under these conditions the LDL receptor is saturated, and cholesterol esterification is a measure of the rate of cholesterol delivery by the LDL-receptor pathway. In other experiments esterification was measured after preincubation in growth medium for 1 h, with or without additions, as specified in the legends to Figures.

Cholesterol esterification was assayed in 35 mm-diam. dishes by measuring the incorporation of [³H]oleate (0.1 mM; sp. activity 150–300 d.p.m./pmol, complexed to BSA as described in [13]) into cellular cholesterol esters. At the end of the assay, the cells

were chilled and washed, lipids were extracted, and 5000 d.p.m. of [^{14}C]cholesterol oleate was added to the extract as a recovery standard [13]. After extraction of lipids, the cell monolayers were allowed to dry, before dissolution in 0.1 M-NaOH and protein assay. Lipid extracts were dried and redissolved in 100 μl of acetone. After 25 μg of cholesterol ester was added as carrier, the lipids were applied to t.l.c. plates (Kieselgel 60, Merck 5553) and separated in heptane/diethyl ether/glacial acetic acid (90:10:1, by vol.). The developed plates were allowed to dry, sprayed with 10% (v/v) H_2SO_4 in methanol and heated gently until the characteristic mauve colour of cholesterol ester was visible. The radioactivity in the stained areas was assayed by scintillation counting.

LDL binding

All experiments were carried out in six-well 35 mm-diam. cluster dishes, with near-confluent cells, unless stated otherwise. In most experiments cultures were washed with Dulbecco's phosphate-buffered saline and incubated with LPDM for 24 h to up-regulate the LDL receptor before addition of the agents. Measurements were carried out up to 24 h after adding the agents. In some cases, after washing the cultures, experiments were initiated by addition of LPDM with or without the specified agents for 24 h before measuring LDL binding.

The amount of ^{125}I -LDL bound by the cells at 4 °C was assayed as described by Goldstein *et al.* [13] in the absence of added agents. The procedures are summarized briefly in [9] and were applied to monolayers of human skin fibroblasts, MRC5 cells and vascular smooth-muscle cells. When LDL was included in the experimental medium (Table 2), cells were incubated with LPDM alone for 30 min at 37 °C to remove bound unlabelled LDL, before measurement of ^{125}I -LDL binding at 4 °C.

Other assays

Cyclic AMP concentrations were determined by competitive binding assay using Amersham kit no. TRK432. Triplicate 35 mm dishes were extracted with ice-cold 65% (v/v) ethanol, and cyclic AMP was assayed in the dried extracts. Protein content was determined by the method of Lowry *et al.* [17], with BSA as standard.

Statistical and kinetic analysis

Student's unpaired *t* test was used to determine the statistical significance of differences between groups. Unless stated otherwise, studies were carried out in triplicate dishes of cells, and results (presented as means $\pm$ s.d.) are typical of at least two independent experiments.

To calculate K_a values, experimental data were fitted to the Michaelis-Menten curve by using a non-linear-regression data-analysis program for the IBM PC (Enzfitter, from R. J. Leatherbarrow, Elsevier Biosoft, Cambridge, U.K.).

RESULTS AND DISCUSSION

Synthesis of functional LDL receptor is stimulated by increased cyclic AMP

Incubation of human vascular smooth-muscle cells for 24 h in LPDM with the cyclic AMP-elevating agents forskolin (100 μM) and CPT-cAMP (600 μM) resulted in increased synthesis of LDL receptor protein (Fig. 1). Receptor synthesis was measured as the amount of [^{35}S]methionine incorporated into the 160 kDa protein, immunoprecipitated by a specific monoclonal antibody to the LDL receptor (IgG-C7 [15]). The incorporation of [^{35}S]methionine into total cell protein was not altered under these conditions (results not shown). In an exactly parallel experiment (Table 1), we observed that the same agents enhanced the LDL-dependent esterification of cholesterol in these cells. In the latter

Fig. 1. Effect of forskolin, CPT-cAMP and LDL on [^{35}S]methionine incorporation into LDL receptor protein over 24 h in cultured human vascular smooth-muscle cells

Cultures were incubated for 24 h in LPDM containing 5 μM [^{35}S]methionine with the following additions: lanes 1 and 4, control; lanes 2 and 6, 100 μM -forskolin; lane 3, 80 μg of LDL/ml; lane 5, 600 μM -CPT-cAMP. Fluorographs of two representative experiments are shown: Expt. A, lanes 1-3; Expt. B, lanes 4-6. See the Materials and methods section for further details. The arrows indicate the position of the 160 kDa LDL receptor protein.

Table 1. LDL-receptor synthesis and function in the presence of CPT-cAMP and forskolin

LDL-receptor synthesis was measured as described in the legend to Fig. 1. LDL-receptor function was assessed by measuring the amount of cholesterol available for esterification. Cultures of human vascular smooth-muscle cells were incubated for 24 h in LPDM in the presence of the specified agents. The cultures were then washed and incubated with 80 μg of LDL/ml for 6 h, followed by measurement of [^3H]oleate incorporation into cholesterol esters, as described in the Materials and methods section. Each value is the mean $\pm$ s.d. of measurements in triplicate dishes: **P* < 0.02 with respect to appropriate controls.

Additions to LPDM	LDL-receptor function (cholesterol esterification; pmol/h per mg of cell protein)		LDL-receptor synthesis (relative densitometry units)	
	Expt. A	Expt. B	Expt. A	Expt. B
None	1480 $\pm$ 172	1484 $\pm$ 148	100	100
CPT-cAMP (600 μM)	—	2290 $\pm$ 348*	—	198
Forskolin (100 μM)	2344 $\pm$ 118*	2070 $\pm$ 232*	250	228
LDL	134 $\pm$ 27	—	10	—

Fig. 2. Effect of forskolin on the time course of [35 S]methionine incorporation into (a) and loss from (b) the LDL receptor protein in cultured human vascular smooth-muscle cells

(a) Cultures were preincubated in LPDM for 24 h, to induce the LDL receptor, followed by fresh LPDM containing 5 μ M-methionine alone (□) or 5 μ M-methionine and 100 μ M-forskolin (■) for the indicated time. [35 S]Methionine was present during the last 4 h of incubation. The results are means $\pm$ S.D. for four separate experiments on cells from three different individuals: ** $P < 0.02$, *** $P < 0.01$ with respect to controls. (b) Cultures were incubated in LPDM containing 5 μ M-[35 S]methionine for 24 h to induce and prelabel the LDL receptor, followed by a cold chase in LPDM containing 500 μ M-methionine alone (□) or 500 μ M-methionine and 100 μ M-forskolin (■) for the indicated times. The LDL receptor protein was immunoprecipitated and the radioactivity of the 160 kDa mature receptor was estimated as described in the Materials and methods section.

experiment it is important to note that cells were preincubated for 24 h with forskolin and CPT-cAMP, which were then removed, before assaying esterification in the presence of a saturating concentration of LDL (see legend to Table 1 for details). Goldstein *et al.* [18] have shown that the LDL-dependent esterification of cholesterol is a measure of the cholesterol-delivering activity of the LDL receptor pathway. Therefore the results presented in Fig. 1 and Table 1 show that increasing cyclic AMP stimulates the labelling of LDL receptors with [35 S]methionine and simultaneously increases cholesterol delivery to the cell from LDL. These findings add to our previous observations where, in the presence of increased cyclic AMP, [125 I]-LDL binding, internalization and degradation were stimulated by a mechanism causing increased numbers of receptors (V_{max}). This stimulation did not occur in the presence of cycloheximide or actinomycin D [9].

The time course of cyclic AMP stimulation of [35 S]methionine incorporation is presented in Fig. 2(a). Smooth-muscle cells were preincubated for 24 h in LPDM to achieve up-regulation of the receptor. The cultures were then incubated with fresh LPDM with or without forskolin and pulsed with [35 S]methionine for 4 h periods over the next 24 h. The incorporation period was short

compared with the $t_{1/2}$ of the LDL receptor ($t_{1/2} = 12$ h [19]). Forskolin stimulation became apparent 12–16 h after addition, when it increased the rate of [35 S]methionine incorporation into the 160 kDa mature receptor protein by 2-fold over the controls (Fig. 2a). The forskolin-induced increase in the rate of LDL-receptor synthesis was still significant at 24 h. The presence of 2.5 μ g of actinomycin D/ml in the incubation medium prevented the stimulation of receptor protein synthesis by forskolin (results not shown). Under identical experimental conditions we found no evidence that forskolin inhibits degradation of prelabelled LDL receptor protein (Fig. 2b) or alters its $t_{1/2}$ of 12 h.

In a parallel experiment with the same smooth-muscle cells, we showed that forskolin treatment increased cyclic AMP from 5.3 ± 2.6 to 97.2 ± 5.3 pmol/mg of cell protein in 4 h, falling to 32.9 ± 2.5 pmol/mg after 24 h. The cyclic AMP-stimulated up-regulation of the LDL receptor is not connected with the growth-related stimulation of receptor-gene expression [20]. Stout [21] has shown that cyclic AMP derivatives inhibit general DNA synthesis in smooth-muscle cells. The data support a specific cyclic AMP-mediated stimulation of the rate of LDL-receptor synthesis, perhaps by a transcriptional mechanism.

As far as we are aware, the effect of cyclic AMP on LDL-receptor protein synthesis has previously been investigated only in specialized steroid-hormone-producing cells. Golos & Strauss [22] reported an increase in LDL-receptor synthesis in human granulosa cells in the presence of 8-bromo cyclic AMP and concluded that the increase was, at least in part, independent of alterations in cellular cholesterol balance. In a later study [23] the same authors reported that increased synthesis of LDL-receptor mRNA in the presence of 8-bromo cyclic AMP was resistant to sterol-mediated feedback inhibition, whereas the stimulatory effect of 8-bromo cyclic AMP on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase mRNA synthesis was over-ridden by sterols. Thus in granulosa cells there is differential regulation of HMG-CoA reductase and LDL-receptor mRNA in the presence of sterol-mediated negative feedback. It remains to be seen whether the control of the synthesis of HMG-CoA reductase by cyclic AMP resembles that of the LDL receptor in smooth-muscle cells.

Stimulation of LDL receptor activity by cyclic AMP is not mediated by altered cholesterol synthesis or esterification

The expression of the LDL receptor in cultured cells is subject to sterol-mediated feedback repression [1]. When cellular cholesterol concentration is decreased, the repression is relieved and LDL receptor activity rises. In the following experiments, we investigated the possibility that cyclic AMP up-regulates the LDL receptor by enhancing enzymic processes which deplete cellular cholesterol.

Phosphorylation of HMG-CoA reductase results in inactivation of the enzyme and rapid suppression of cellular cholesterol synthesis in a variety of tissues and cultured cells [24,25]. The increased cellular cyclic AMP concentration generated by forskolin might initiate inactivation of the enzyme, decreasing cellular cholesterol concentration and consequently increasing LDL-receptor expression. We used 2 μ M-mevinolin, a specific inhibitor of HMG-CoA reductase [14], to cause > 95% inhibition of cholesterol synthesis in human vascular smooth-muscle cells which had been depleted of cholesterol in LPDM for 24 h. Under these conditions, mevinolin enhanced specific LDL binding by 40%, and forskolin increased this by a further 80% (Table 2). The effects of forskolin and mevinolin were more than additive. Since cholesterol synthesis was substantially inhibited by mevinolin alone, the effect of forskolin is unlikely to be mediated by further inhibition of HMG-CoA reductase. Therefore stimulation of the LDL receptor activity by forskolin is not

Table 2. Cyclic AMP-elevating agents up-regulate LDL-receptor expression in the presence of inhibitors of cholesterol synthesis (mevinolin) and esterification (58035)

Cultures were preincubated for 24 h in LPDM, to induce the LDL receptor, followed by another 24 h in fresh LPDM with or without the specified additions. At the end of the incubation ^{125}I -LDL binding was measured in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean $\pm$ S.D. for the number of determinations given in parentheses: * $P < 0.02$, ** $P < 0.01$ with respect to appropriate controls. The data shown are typical of four independent experiments in which cyclic AMP-elevating agents stimulated ^{125}I -LDL binding by an average of 77 % without effect of 58035, whereas mevinolin enhanced the forskolin effect by an average of 73 %.

Additions	LDL binding (ng/mg of cell protein)			
	None	Forskolin (100 μM)	Dideoxyforskolin (100 μM)	CPT-cAMP (100 μM)
None	60 $\pm$ 11 (6)	125 $\pm$ 16 (7)**	60 $\pm$ 4 (4)	82 $\pm$ 4 (3)*
Mevinolin (2 μM)	102 $\pm$ 6 (4)**	184 $\pm$ 14 (4)**	—	—
58035 (2 μM)	40 $\pm$ 7 (3)	116 $\pm$ 8 (3)**	—	86 $\pm$ 4 (3)**

Fig. 3. Effect of forskolin on LDL binding to human vascular smooth-muscle cells (a) and human MRC5 lung fibroblasts (b), in the presence of 25-hydroxycholesterol

Cultures were preincubated for 24 h in LPDM, to induce the LDL receptor, followed by another 24 h in fresh LPDM containing increasing concentrations of 25-hydroxycholesterol without (□) or with (■) 100 μM -forskolin. At the end of the incubation LDL binding was measured in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean $\pm$ S.D. of measurements in triplicate dishes: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ with respect to controls. The results depicted are typical of four independent experiments in which forskolin stimulated ^{125}I -LDL binding by 54–165 % at 1 μg of 25-hydroxycholesterol/ml, all effects being statistically significant.

secondary to inhibition of HMG-CoA reductase. Note that dideoxyforskolin, an analogue of forskolin which does not activate adenylate cyclase [26], did not alter LDL binding (Table 2).

We have shown that independent changes in ACAT activity can influence the expression of the LDL receptor in human skin fibroblasts [27] and rat hepatocytes [28]. The activity of ACAT may be controlled by cyclic AMP via phosphorylation, but the effect is tissue-dependent. The enzyme is activated by

phosphorylation in liver and the intestinal mucosa, but is inactivated in the adrenal [29]. In order to test the possibility that the action of cyclic AMP-elevating agents on LDL receptor activity is mediated by activation of ACAT, we carried out experiments in the presence of compound 58035, a potent and specific inhibitor of this enzyme [30]. Table 2 shows that the stimulatory effects of forskolin and CPT-cAMP on LDL binding were unaffected by the presence of 2 μM -58035, a concentration which we have found to abolish cholesterol esterification in these cells (results not shown).

We conclude that cyclic AMP-dependent up-regulation of the LDL receptor is not mediated by alteration in cholesterol concentrations through primary changes in HMG-CoA reductase or ACAT activity.

Cyclic AMP up-regulation of the LDL receptor is maintained in the presence of oxysterol repression

Feedback suppression of the LDL receptor is thought to be mediated not by the cholesterol molecule itself but by an oxidized metabolite resembling the potent synthetic repressor 25-hydroxycholesterol [31,32]. In human smooth-muscle cells (Fig. 3a) and lung fibroblasts (Fig. 3b), 25-hydroxycholesterol repressed LDL receptor activity in the presence and absence of forskolin. Over the range of 25-hydroxycholesterol concentrations studied here, LDL binding in the presence of forskolin was always about 2-fold higher than at the same repressor concentration without forskolin. However, at high hydroxysterol concentration, forskolin did not return the LDL receptor activity to the level in control cells incubated without 25-hydroxycholesterol. Thus in these cells cyclic AMP was not as potent at up-regulating the LDL receptor as in steroid-synthesizing granulosa cells, where cyclic AMP could over-ride sterol-mediated negative feedback [22].

Cyclic AMP increases cellular cholesterol pools

The rate of esterification of cellular cholesterol mainly reflects the size of the pool of cholesterol substrate for ACAT [16a]. Cultures of smooth-muscle cells in normal, LDL-containing, growth medium incorporated [^3H]oleate into cholesterol esters at a rate of 400 pmol/h per mg of cell protein. After exposure to 100 μM -chloroquine for 1 h, the rate of incorporation was decreased to 50 pmol/h per mg. Chloroquine is a known inhibitor of lysosomal function in the LDL pathway [33,34]. Its rapid inhibition of cholesterol esterification is compatible with the notion that most of the cholesterol used in this process is derived from lysosomal hydrolysis of LDL-borne cholesterol esters.

The addition of CPT-cAMP to the LDL-containing medium for 1 h rapidly increased the rate of esterification in the absence

Fig. 4. Effect of CPT-cAMP on the esterification of LDL cholesterol and non-lipoprotein cholesterol in cultured human vascular smooth-muscle cells

(a) Cultures in growth medium (containing LDL) were incubated for 1 h with increasing concentrations of CPT-cAMP, in the absence (□) or presence (■) of 100 μM-chloroquine, before measurement of cholesterol esterification (as described in the Materials and methods section). Each value is the mean ± s.d. of measurements in triplicate dishes, and the data shown are typical of four independent experiments in which 250 μM-CPT-cAMP stimulated cholesterol esterification by 58–68 %, all effects being statistically significant at $P < 0.05$. (b) Cultures were preincubated for 24 h with LPDM to deplete cellular cholesterol. Increasing concentrations of free cholesterol in serum-free DMEM containing 5 mg of BSA/ml were added for 24 h. For the last 1 h of this incubation, the medium was replaced with fresh cholesterol-containing medium without (○) or with (●) 300 μM-CPT-cAMP. After 1 h, 100 μM- ^3H oleate was added to determine the rate of cholesterol esterification. Each value is the mean ± s.d. of measurements in triplicate dishes, and the results depicted are typical of three independent experiments in which CPT-cAMP caused no significant stimulation of esterification of exogenous cholesterol.

of chloroquine. However, CPT-cAMP did not alter the low rate of esterification observed in the presence of chloroquine (Fig. 4a), suggesting that the stimulation of esterification by CPT-

cAMP is mediated via a lysosomal process and does not occur when lysosomal function is impaired by chloroquine. It is important to note that the effect of CPT-cAMP on the rate of esterification is rapid and observed within 1 h of exposure. This is in contrast with the effects of CPT-cAMP and forskolin on the LDL receptor, which occur only after 12–24 h exposure ([9] and Fig. 2a).

In cells depleted of LDL cholesterol in LPDM, the low basal rate of esterification was increased by up to 24-fold by adding exogenous unesterified non-lipoprotein cholesterol; the rates were not altered in the presence of CPT-cAMP (Fig. 4b). Chloroquine had no effect on the rate of esterification of exogenous unesterified non-lipoprotein cholesterol (results not shown), indicating that there is no lysosomal involvement in these circumstances.

There is evidence that the lysosomal process stimulated by cyclic AMP could be the hydrolysis of LDL-borne cholesterol esters. Hajjar [10] has demonstrated increased activities of the lysosomal cholesterol esterase (EC 3.1.1.13) in homogenates prepared from smooth-muscle cells which were preincubated briefly with agents that increase cyclic AMP. The data presented in Fig. 4 are compatible with elevated cyclic AMP accelerating lysosomal hydrolysis of LDL-borne cholesterol esters, thus increasing cellular cholesterol pools and consequently the supply of free cholesterol for esterification (Fig. 4a). The availability of non-LDL exogenous free cholesterol for esterification is independent of the activity of cholesterol esterases, and therefore is not affected by cyclic AMP (Fig. 4b).

It is of interest that cyclic AMP had no direct effect on the rate of cholesterol esterification in human vascular smooth-muscle cells (Fig. 4b). The stimulation observed in Fig. 4(a) was due to an increased availability of cholesterol, and not a direct effect on the esterification reaction itself. This was in contrast with findings reported in the adrenal, where elevated cyclic AMP inhibited esterification [29].

Dual effect of cyclic AMP on the activity of the LDL receptor

We have demonstrated so far that exposure of cultured human vascular smooth-muscle cells to cyclic AMP for 12–24 h results in increased synthesis of LDL receptor protein (Figs. 1 and 2). We have also shown that a short exposure for 1 h only increases the amount of cellular cholesterol measured by its availability for esterification (Fig. 4). It is well established that elevated cellular cholesterol concentrations repress the activity of the LDL receptor [1]. So cyclic AMP could have two opposing effects on LDL receptor function: a direct stimulatory effect on LDL-receptor synthesis and an indirect, cholesterol-mediated, in-

Table 3. Effects of forskolin and chloroquine on ^{125}I -LDL binding to human skin fibroblasts, in the presence and absence of LDL

Cultures of human skin fibroblasts were preincubated with LPDM for 24 h, followed by another 24 h in fresh LPDM with additions as specified below. At the end of the incubation, the cultures were washed and incubated with LPDM alone for 30 min at 37 °C, to remove unlabelled LDL bound to cell-surface receptors. ^{125}I -LDL binding was then assayed in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean ± s.d. of measurements in triplicate dishes: * $P < 0.05$, *** $P < 0.01$ column C versus column A; ††† $P < 0.01$ column D versus column B. The data shown are typical of three independent experiments performed with LDL at 2.1–85.0 μg/ml in which forskolin stimulation was enhanced with chloroquine by 52–300 %.

Additions ... [LDL] (μg/ml)	LDL binding (ng/mg of cell protein)			
	None (A)	Chloroquine (100 μM) (B)	Forskolin (100 μM) (C)	Chloroquine plus forskolin (D)
0.0	64 ± 2.6	88 ± 1.3	145 ± 14***	351 ± 25†††
3.4	23 ± 1.5	76 ± 6.0	50 ± 12*	287 ± 31†††
85.0	5 ± 1.0	44 ± 0.5	7 ± 1.7	166 ± 26†††

Fig. 5. Chloroquine enhances the stimulation of LDL-receptor up-regulation by forskolin in human vascular smooth-muscle cells

Cultures were preincubated for 24 h in LPDM, to induce the LDL receptor, followed by another 24 h in fresh LPDM containing increasing concentrations of forskolin without (□) or with (■) 100 μM -chloroquine. At the end of the incubation, LDL binding was measured in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean $\pm$ S.D. of measurements from three independent experiments. All effects of forskolin and chloroquine are statistically significant ($P < 0.01$).

hibitory effect. The following experiments were designed to test this hypothesis.

Incubation of human skin fibroblasts with chloroquine for 24 h attenuated the down-regulation of the LDL receptor by LDL (Table 3, column B versus A). Chloroquine was very effective at counteracting feedback repression at high LDL concentrations, increasing binding 3.5- and 9-fold in the presence of 3.4 and 85 μg of LDL/ml respectively (Table 3, column B versus A). This attenuation is compatible with inhibition of lysosomal release of repressive cholesterol from LDL-borne cholesterol ester in the presence of chloroquine [34], thus decreasing feedback inhibition of the LDL receptor. Forskolin also attenuated the repressing effect of low LDL concentrations (Table 3, column C versus A), but at high concentrations of LDL (85 μg /ml) all such effects of forskolin were lost. In the presence of both chloroquine and forskolin, LDL binding was substantially higher than in the presence of each agent alone (Table 3, column D versus B or C). In both the absence and the presence of added LDL, chloroquine enhanced the stimulation of LDL receptor activity by forskolin, so that the effects of the two agents were much more than additive. The forskolin stimulation was now seen even in the presence of 85 μg of LDL/ml.

The data presented in Table 3 are consistent with two effects of forskolin via elevated cyclic AMP: (1) stimulation of LDL receptor function, owing to increased receptor synthesis (Figs. 1 and 2); (2) repression of LDL receptor function by the increase in the supply of cholesterol, owing to accelerated lysosomal processing of LDL-borne cholesterol ester. In the presence of chloroquine, lysosomal hydrolysis of cholesterol esters and subsequent suppression of the LDL receptor are inhibited, so that stimulation of LDL-receptor synthesis operates unopposed.

Fig. 5 shows the effect of 100 μM -chloroquine on forskolin-mediated up-regulation of the LDL receptor activity in vascular smooth-muscle cells exposed to chloroquine and/or forskolin for

24 h. In this experiment no LDL was added to the cells, which had been depleted of cholesterol by 24 h incubation in lipoprotein-deficient serum, before addition of chloroquine or forskolin. Forskolin increased LDL receptor activity in a concentration-dependent manner ($K_a = 49 \mu M$). However, the stimulation was more pronounced in the presence of chloroquine, and the two compounds acted synergistically to increase LDL receptor activity (Fig. 5). Thus 20 μM -forskolin alone increased LDL receptor activity by 55%, and 100 μM -chloroquine alone increased LDL receptor activity by 36%. However, in the presence of 20 μM -forskolin and 100 μM -chloroquine, the effects were more than additive, with 139% increase in LDL receptor activity. The presence of chloroquine increased the sensitivity of the response of the LDL receptor to forskolin, decreasing the K_a from 49 μM to 15 μM . The calculated maximum binding of LDL to its receptor was increased by chloroquine from 160 ng/mg to 267 ng/mg.

It is of interest that addition of chloroquine to cultures depleted of cholesterol caused a 38% ($P < 0.01$) increase in LDL binding (Fig. 5 and Table 3). This indicates that, even without added LDL and after 24 h pretreatment with LPDM, some lysosomal cholesterol ester stores were still present and, in the absence of chloroquine, were being hydrolysed, yielding free cholesterol, which prevented full expression of the LDL receptor. Similar results were observed when chloroquine was replaced by another inhibitor of lysosomal function, NH_4Cl (results not shown), thus providing further support for lysosomal involvement.

As mentioned above, cholesterol-mediated feedback repression of the LDL receptor is thought to be exerted by an oxidized metabolite of cholesterol [31]. Generation of the presumed oxidized sterol repressor molecule is inhibited by ketoconazole [35]. We found that addition of 50 μM -ketoconazole to human smooth-muscle cells (Fig. 6a) or human lung fibroblasts (Fig. 6b) enhanced LDL binding significantly even after 24 h preincubation in LPDM, confirming our suggestion (above) that this medium did not remove all repressing cholesterol from the cells. When forskolin (Fig. 6b) or permeant cyclic AMP derivatives (Fig. 6a) were added to cells together with ketoconazole, the LDL receptor activity was increased more than additively.

These observations complement the findings obtained with chloroquine (Fig. 5 and Table 3), showing that inhibition of lysosomal processing of LDL or inhibition of oxysterol formation enhance or 'unmask' the stimulatory action of cyclic AMP on the expression of the LDL receptor in human extrahepatic cells.

If the observed response of LDL receptor activity to cyclic AMP is a sum of the inhibition due to increased hydrolysis of cholesterol esters and the stimulation due to increased expression of the LDL-receptor gene, then the contribution of each of these processes could differ between cell types, cell lines, and even within the same cell line, depending on passage number etc. This could account for the conflicting reports about interaction between cyclic AMP and the LDL receptor, referred to in the Introduction to this paper. We have carried out numerous experiments examining these variables using human skin fibroblasts, smooth-muscle cells and the human foetal-lung fibroblast cell line MRC5. In all these experiments chloroquine unmasked or enhanced the underlying stimulatory effect of cyclic AMP on LDL receptor activity. The effect was independent of degree of cell confluence. Fig. 7 shows two representative cases. The MRC5 cells used in Fig. 7(a) have never, in our experiments, shown the inhibition of LDL receptor activity by cyclic AMP reported in the same cells by Maziere *et al.* [7]. The cells consistently responded to added cyclic AMP derivatives with little or no stimulation, whereas forskolin caused significant increase in LDL binding. However, in the presence of

Fig. 6. Enhancement by ketoconazole of cyclic-AMP-mediated stimulation of LDL binding to human vascular smooth-muscle cells (a) and human MRC5 lung fibroblasts (b)

Cultures were preincubated for 24 h in LPDM, to induce the LDL receptor, followed by another 24 h in fresh LPDM without or with 50 μM-ketoconazole, and the following additions: control (□); 100 μM-forskolin (■); 1 mM-db-cAMP (▨); 600 μM-CPT-cAMP (▩). At the end of the incubation LDL binding was measured in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean ± S.D. of measurements in triplicate dishes: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ with respect to controls. The results depicted are typical of six independent experiments in which ketoconazole enhanced the stimulatory effects of cyclic AMP-elevating agents on 125 I-LDL binding by 29–156 %, all effects being statistically significant.

chloroquine, all cyclic AMP-elevating agents stimulated LDL binding. Fig. 7(b) shows one of our own smooth-muscle cell lines which, only at high passage (> 30), began to show inhibition of LDL receptor activity with added cyclic AMP and forskolin. In the presence of chloroquine the inhibitory response was removed, revealing the 1.5–2.5-fold stimulation of LDL receptor activity by the cyclic AMP-elevating agents that we had previously reported in the same cells at low passage [9]. This is evidence for a lysosomal involvement in the inhibition of LDL receptor activity by added cyclic AMP derivatives. It also strongly supports the dual-role hypothesis for the actions of cyclic AMP on expression of the LDL receptor, as advanced above.

The responses of cholesterol metabolism in the cell to increased cyclic AMP can be regarded as a consistent set of actions which ensure a continuous supply of cholesterol for cellular use, in the short term by increased hydrolysis of cholesterol esters and in the longer term by stimulating the synthesis of the LDL receptor. We expect the net effect of cyclic AMP elevation in a cell to be dependent on the cell type, to the extent that this determines relative sizes of lysosomal and regulatory pools of cholesterol. In fast-growing cells or cells with rapid cholesterol metabolism or a pathway for its secretion, the up-regulating response would be expected to dominate.

In conclusion, these results show that cyclic AMP-elevating agents further increase LDL-receptor synthesis and activity in extrahepatic cells in which LDL-receptor synthesis was

Fig. 7. 'Unmasking' by chloroquine of cyclic-AMP-mediated stimulation of LDL binding to human MRC5 lung fibroblasts (a) and highly passaged vascular smooth-muscle cells (b)

Cultures were preincubated for 24 h in LPDM, to induce the LDL receptor, followed by another 24 h in fresh LPDM with or without chloroquine and the following additions: control (□); 1 mM-db-cAMP (▨); 600 μM-CPT-cAMP (▩); 100 μM-forskolin (■). At the end of the incubation, LDL binding was measured in the absence of added agents at 4 °C (see the Materials and methods section). Each value is the mean ± S.D. of measurements in triplicate dishes: * $P < 0.001$ with respect to controls. These results are typical of ten independent experiments in which cyclic AMP-elevating agents, in the presence of chloroquine, increased the binding of 125 I-LDL by 71–277 %, all effects being statistically significant.

maximally induced (a) by removal of sterol and (b) by prevention of sterol formation, sterol supply or sterol repression. This constitutes evidence for a cyclic AMP-mediated, sterol-independent, control of LDL-receptor synthesis.

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