Calcium-antagonists inhibit secretion of very-low-density lipoprotein from cultured rat hepatocytes

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The effects of different calcium-antagonists on secretion of very-low-density lipoprotein (VLDL) from cultured rat hepatocytes were examined. Verapamil (an inhibitor of voltage-dependent calcium channels) and EGTA (a calcium chelator) decreased VLDL-triacylglycerol secretion in a concentration-dependent manner, with maximum inhibition (about 90 %) at 0.2 mM-verapamil and 5 mM-EGTA. Inorganic calcium-antagonists such as lanthanum, nickel, cobalt and manganese decreased secretion of VLDL-triacylglycerol by 55–95 %, whereas the calcium-agonist barium did not affect secretion. Inhibition of VLDL-triacylglycerol secretion appeared within 30 min, without inhibition of triacylglycerol synthesis. Pulse-chase experiments revealed that verapamil and cobalt inhibited the secretory pathway itself. Cobalt showed a concentration-dependent inhibition of VLDL-triacylglycerol secretion, with maximal effect at 8 mM. Although inhibition by cobalt was not completely reversible, Trypan Blue exclusion and lactate dehydrogenase leakage indicated that the hepatocytes were not injured by cobalt or any of the other calcium-antagonists tested. Inhibition of protein synthesis by cycloheximide did not affect triacylglycerol secretion (up to 2 h), and the observed effects were therefore probably not due to impaired production of apolipoproteins. Taken together, these results suggest that calcium is important for secretion of VLDL particles.

INTRODUCTION

Calcium is an important regulator of several cellular processes (Bolton, 1979; Cheung, 1980; Rasmussen, 1986; Williamson *et al.*, 1981), especially as a trigger to release many cellular products (Botelho & Dartt, 1980; Eto *et al.*, 1974; Hagaiwara & Byerly, 1981; Russell & Thorn, 1974). Calcium is liberated from intracellular stores (Botelho & Dartt, 1980; Rasmussen, 1986) or enters the cytoplasm from the extracellular space through receptor-operated or voltage-dependent channels in the plasma membrane (Hagaiwara & Byerly, 1981; Rasmussen, 1986). Calcium may be involved in secretion through one or more of at least three processes: (1) synthesis of secretory products, (2) regulators of microtubuli and microfilaments, which is crucial for transport of secretory products to the plasma membrane, or (3) fusion between secretory vesicles and the plasma membrane, by neutralizing electronegative charges and collapsing an energy barrier (Malaisse, 1984).

In the present study we report the effects of several calcium-antagonists on secretion of VLDL from rat hepatocytes. Among the agents studied are verapamil, EGTA and several different cations. Verapamil is one of several drugs which binds to the voltage-dependent channels and thereby inhibits passage of calcium through the plasma membrane (Henry, 1980; Nachshen & Blaustein, 1979). It has been shown that verapamil inhibits secretion from different types of cells (Botelho & Dartt, 1980; Eto *et al.*, 1974; Malaisse, 1984; Russell & Thorn, 1974). EGTA is a calcium chelator which binds calcium and inhibits several calcium-dependent processes, including secretion of hormones and lacrimal liquid (Botelho & Dartt, 1980; Hopkins & Walker,

1978). Cobalt probably can bind to calcium channels as well as to intracellular calcium-binding sites (Kotsias *et al.*, 1986; Meyer & Cooper, 1983; Thaw *et al.*, 1984; Triggle & Swamy, 1980; Wollheim & Janjic, 1984) and thereby interfere with calcium-dependent processes.

We examined the effect of these agents on triacylglycerol secretion in cultured rat hepatocytes. Previous reports have shown that these cells secrete triacylglycerol at rates comparable with those observed *in vivo* (Davis *et al.*, 1979; Drevon *et al.*, 1980), and calcium currents across the plasma membrane of cultured hepatocytes have been demonstrated (Altin & Bygrave, 1985; Chen *et al.*, 1978).

MATERIALS AND METHODS

Materials

Arginine-free DME medium, gentamicin and newborn-calf serum were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. The medium was supplemented with ornithine (0.5 mM), Hepes (10 mM) and NaHCO₃ (24 mM) from Sigma Chemical Co., St. Louis, MO, U.S.A. [2-³H]Glycerol (200 Ci/mol) was purchased from New England Nuclear, Dreieich, Germany. Collagenase (type I), lactate dehydrogenase reagent kit, bovine insulin, EGTA, CoCl₂ and the other inorganic salts (as chlorides), oleic acid and BSA (essentially fatty acid-free) were obtained from Sigma. Verapamil was delivered by Meda A/S, Oslo, Norway. Culture dishes (60 mm diam.) were purchased from Costar, Cambridge, MA, U.S.A. All other chemicals and solvents were high-purity commercial materials.

Abbreviations used: VLDL, very-low-density lipoprotein; DME medium, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

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Preparation of cultured hepatocytes

Hepatocytes were prepared from male albino rats (200–300 g) by collagenase perfusion and plated as described elsewhere (Nossen *et al.*, 1984).

Experiments with hepatocytes

After overnight incubation, the cells were changed to a serum-free DME medium or a carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM) and Hepes (10 mM), supplemented with the components indicated in the legends to Tables and Figures. Preliminary experiments showed that synthesis and secretion of triacyl- ^3H glycerol were similar in DME medium, containing 1.8 mM-calcium, and in carbonate-free buffer without calcium, indicating that the cellular content of calcium was sufficient for synthesis and secretion of triacyl- ^3H glycerol up to 3 h (results not shown). DME medium with 1.8 mM-calcium was used in experiments with verapamil, since this drug may act as an inhibitor of calcium influx. The inorganic cations form insoluble carbonates, and therefore medium without carbonate was chosen for the experiments with these antagonists and with EGTA.

All drugs, except EGTA, were added to the hepatocytes from fresh aqueous stock solutions. Addition of EGTA to the medium caused pH changes, and this agent was therefore added to the medium and pH was adjusted before the medium was given to the cells. EGTA also decreased attachment of hepatocytes to the dishes, and it was impossible to test if inhibition of triacylglycerol secretion by this compound was reversible in plated cells. Thus reversibility of EGTA was tested in suspended hepatocytes (Drevon *et al.*, 1977).

Determination of VLDL-triacylglycerol

After incubation, medium and cells were separated and analysed for triacylglycerol. Triacyl ^3H glycerol was determined by extraction, isolation by t.l.c. and liquid-scintillation counting (Nossen *et al.*, 1984). The mass of secreted triacylglycerol was determined by an enzymic method for free glycerol (fluorimetry) (Wieland, 1974). VLDL-triacylglycerol secretion was determined by incorporation of ^3H glycerol into triacylglycerol recovered in the medium. After preincubation of cells with this label for 30 min, secretion of VLDL-triacylglycerol was linear with time for at least 2 h and more than 95% of triacyl ^3H glycerol in the medium appeared in the VLDL fraction ($d < 1.006$) (Nossen *et al.*, 1984). The rate of appearance of triacyl ^3H glycerol in the medium was therefore taken as a satisfactory estimate for secretion of newly synthesized VLDL-triacylglycerol.

Protein content of the dishes was determined with BSA as standard (Lowry *et al.*, 1951).

Determination of lactate dehydrogenase leakage and Trypan Blue exclusion

After incubation of cultured hepatocytes, media were collected and lactate dehydrogenase was assayed by a colorimetric method, by using a diagnostic kit supplied by Sigma. Viability was determined by Trypan Blue exclusion by suspended hepatocytes, because of technical difficulties with counting of plated cells.

Presentation of data

All results are presented as means $\pm$ S.D. for triplicate dishes and represent at least three separate experiments.

Fig. 1. Dose-response of verapamil on secretion of triacylglycerol

Hepatocytes were incubated in DME medium containing oleic acid (1.0 mM), BSA (0.13 mM) and ^3H glycerol (5 $\mu\text{Ci}/\text{ml}$, 25 μM). After 30 min preincubation, different concentrations of verapamil were added and the cells incubated for 2 h.

Counting efficiency for ^3H was about 55%, and quenching was similar for all agents tested.

RESULTS

Inhibition of triacylglycerol secretion by organic calcium-antagonists

Verapamil inhibited triacylglycerol secretion in a concentration-dependent manner. Maximum inhibition was reached at 200 μM -verapamil, which decreased secretion by 90% of control; 12.5 μM and 25 μM of the drug inhibited secretion by 30 and 35% respectively (Fig. 1). Although the inhibition was smaller, measurement of triacylglycerol mass verified these observations. At 200 μM -verapamil, triacylglycerol secretion was decreased by 50%, from 11.2 ± 0.7 to 5.6 ± 1.9 nmol of triacylglycerol/mg of cell protein after 4 h incubation, which may indicate a preferential effect on newly synthesized triacylglycerol.

The inhibitory effect of verapamil on triacylglycerol secretion was apparent within 30 min (Figs. 2a and 2c) and independent of triacylglycerol synthesis (Fig. 2c). Verapamil did not decrease cellular triacylglycerol synthesis (Fig. 2b), and the inhibition of triacylglycerol secretion could be reversed after removal of the drug (Table 1).

The release of triacylglycerol was decreased by 60 and 90% of control at 1 mM- and 5 mM-EGTA respectively (Fig. 3). The inhibitory effect appeared immediately after the linear phase of secretion was reached (Fig. 4a), whereas synthesis of triacylglycerol was unaffected (Fig. 4b).

Fig. 2. Effect of verapamil on secretion and synthesis of triacylglycerol

Hepatocytes were incubated in DME medium containing oleic acid (1.0 mM), BSA (0.13 mM) and [3 H]glycerol (5 μ Ci/ml, 25 μ M). Panels (a) and (b): after 30 min preincubation without verapamil, the cells were incubated for indicated time periods without (○) or with (●) 200 μ M-verapamil. Panel (c): the cells were pulsed with radioactive glycerol for 1 h before they were washed twice and chased in identical medium containing unlabelled glycerol (1 mM), without (○) or with (●) 150 μ M-verapamil.

Inhibition of triacylglycerol secretion by inorganic cations

Manganese, cobalt and nickel (final concn. 4 mM) inhibited secretion of triacylglycerol by 55, 70 and 80 % of control respectively (Fig. 5). The inhibition was even stronger by 4 mM-lanthanum (tervalent), which decreased release of triacylglycerol by 95 % of control. In contrast with the above ions, barium did not affect triacylglycerol secretion (Fig. 5). None of the cations tested decreased synthesis of triacylglycerol (Fig. 5).

Effects of cobalt on triacylglycerol secretion

Since cobalt is a frequently used calcium-inhibitor, the effect of this cation was studied in some detail. Cobalt decreased triacylglycerol secretion in a concentration-dependent manner, with maximum inhibition at 8 mM, at which concentration secretion was 25 % of

Table 1. Reversibility of the inhibitory effects of verapamil, EGTA and cobalt on VLDL-triacylglycerol secretion

Hepatocytes were changed to DME medium (verapamil) or carbonate-free buffer (EGTA and cobalt) containing oleic acid (1.0 mM), BSA (0.13 mM), [3 H]glycerol (5 μ Ci/ml, 25 μ M) and verapamil (100 μ M), EGTA (10 mM) or cobalt (4 mM), and preincubated for 60 min. Then the media were withdrawn and the cells washed twice at 37 °C for 5 min and then re-incubated for 2 h in identical medium with or without the agents added. Control values were verapamil 6329 ± 635 and 6279 ± 320 , EGTA 1637 ± 397 and 2738 ± 722 and cobalt 8987 ± 1078 and 5945 ± 1050 c.p.m./mg of cell protein for preincubation and re-incubation periods respectively. Studies of reversibility of EGTA were performed on suspended hepatocytes.

Agent added	Secretion (% of control)		
	Secretion during preincubation	Secretion during re-incubation	
		With antagonist	Without antagonist
Verapamil (100 μ M)	50 ± 4	23 ± 3	80 ± 12
EGTA (10 mM)	19 ± 2	3 ± 1	30 ± 8
Cobalt (4 mM)	29 ± 6	17 ± 1	32 ± 7

Fig. 3. Dose-response of EGTA on secretion of triacylglycerol

Hepatocytes were incubated in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM), oleic acid (1.0 mM), BSA (0.13 mM), [3 H]glycerol (5 μ Ci/ml, 25 μ M) and indicated concentrations of EGTA for 2 h.

control (Fig. 6). Determination of the mass of secreted triacylglycerol at 4 mM-cobalt showed 45 % inhibition, from 7.3 ± 0.3 to 4.0 ± 0.7 nmol of triacylglycerol/mg of cell protein during 4 h incubation. The inhibitory effect of cobalt on triacylglycerol secretion appeared within 30 min, with no inhibitory effect on triacylglycerol

Fig. 4. Effect of EGTA on secretion (a) and synthesis (b) of triacylglycerol

Hepatocytes were incubated in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM), oleic acid (1.0 mM), BSA (0.13 mM), [^3H]glycerol (5 $\mu\text{Ci/ml}$, 25 μM), without (○) or with (●) EGTA (10 mM) for the indicated time periods.

Fig. 5. Effect of inorganic cations on secretion (a) and synthesis (b) of triacylglycerol

Hepatocytes were incubated in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM), oleic acid (1.0 mM), BSA (0.13 mM) and [^3H]glycerol (5 $\mu\text{Ci/ml}$, 25 μM). After 30 min preincubation, different inorganic cations were added (final concn. 4 mM), and the cells were incubated for 2 h.

Table 2. Trypan Blue exclusion and lactate dehydrogenase leakage from hepatocytes

All values are expressed as % of control. For Trypan Blue exclusion isolated hepatocytes were incubated for 3 h at 37 °C in a shaking water bath in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM) (control medium) and the agents indicated. The dye exclusion by control hepatocytes was $93 \pm 2\%$. For lactate dehydrogenase leakage plated hepatocytes were changed to medium identical with the above and incubated for 3 h before enzyme leakage was assayed. Control values were 668 ± 30 Berger-Broida units (Cabaud & Wroblewski, 1958)/ml per mg of cell protein. Lanthanum promoted precipitation of Trypan Blue, and therefore determination of dye exclusion was impossible. No effect of lanthanum on lactate dehydrogenase activity *in vitro* could be detected.

Agent added ...	Calcium (4 mM)	Verapamil (0.2 mM)	EGTA (5 mM)	Barium (4 mM)	Cobalt (4 mM)	Manganese (4 mM)	Nickel (4 mM)	Lanthanum (4 mM)
Trypan Blue exclusion	98 ± 3	99 ± 5	95 ± 3	100 ± 4	96 ± 6	95 ± 2	101 ± 4	—
Lactate dehydrogenase leakage	98 ± 8	105 ± 4	112 ± 6	99 ± 5	91 ± 8	98 ± 9	82 ± 2	64 ± 5

Fig. 6. Dose-response of cobalt on secretion of triacylglycerol

Hepatocytes were incubated in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM), oleic acid (1.0 mM), BSA (0.13 mM) and [³H]glycerol (5 μ Ci/ml, 25 μ M). After 30 min preincubation, different concentrations of cobalt were added and the cells were incubated for 2 h.

synthesis (Fig. 7). Rather, the synthesis of triacylglycerol in the presence of cobalt was increased (Figs. 5 and 7b). Further, pulse-chase experiments showed that inhibition occurred on the secretory process itself (Fig. 7c). Hepatocytes incubated in the presence of cobalt (4 mM) for 60 min had their triacylglycerol secretion decreased by 71% of control (Table 1). After washing and re-incubation in cobalt-free medium, these cells doubled their secretory rate as compared with cells re-incubated with cobalt, but they did not recover the secretory rate of control cells (Table 1). However, the 'viability' of cells incubated in the presence of 4 mM-cobalt for 3 h was not affected. Both the Trypan Blue exclusion test and leakage of lactate dehydrogenase to the medium (Table 2) indicated that cells exposed to calcium-antagonists were as viable as control cells.

Effect of cycloheximide on triacylglycerol secretion

Cycloheximide (20 μ M) decreased protein synthesis in cultured hepatocytes by 90% after 2 h of incubation (Nossen *et al.*, 1984). Cycloheximide had little effect on the initial secretion of triacylglycerol, but after 2 h of incubation secretion stopped (Fig. 8). Thus continuous protein synthesis was not necessary for triacylglycerol secretion during short-term experiments, up to 2 h.

DISCUSSION

All the calcium-antagonists tested in this study have a marked inhibitory effect on triacylglycerol secretion by cultured rat hepatocytes. We demonstrated that this could not be explained by decreased synthesis of triacylglycerol or apolipoproteins, but by direct inhibitory effect on secretion itself. It has previously been shown that secretion of hormones, neurotransmitters and lacrimal liquid depend on Ca²⁺ ions (Douglas, 1974; Hagaiwara & Byerly, 1981; Malaisse, 1984). However,

Fig. 7. Effect of cobalt on secretion and synthesis of triacylglycerol

Hepatocytes were incubated in carbonate-free buffer containing NaCl (150 mM), KCl (6.7 mM), Hepes (10 mM), oleic acid (1.0 mM), BSA (0.13 mM) and [³H]glycerol (5 μ Ci/ml, 25 μ M). Panels (a) and (b): after 30 min preincubation without cobalt, the cells were re-incubated without (○) or with (●) cobalt (4 mM) for the indicated time periods. Panel (c): after 1 h pulse period with radioactive glycerol, the cells were washed twice, and then chased in identical medium with added glycerol (1 mM), without (○) or with (●) 4 mM-cobalt.

these non-hepatic cells are either excitable (Hagaiwara & Byerly, 1981) or secretory cells responding to special signal molecules, as shown for insulin (Botelho & Dartt, 1980) and luteinizing hormone (Hopkins & Walker, 1978). These cells have in common that their secretory products are released in a burst. Contrarily, secretion of VLDL by hepatocytes is constitutive, i.e. VLDL is continuously released. No specific stimulators of VLDL secretion in hepatocytes are known. The most important regulator of VLDL secretion is the availability of fatty acids via synthesis of triacylglycerol within the cells (Davis & Boogaerts, 1982; Kohout *et al.*, 1971; Nossen *et al.*, 1986). Our data demonstrate that hepatocytes with a constitutive secretory pathway depend on calcium, at least for VLDL secretion.

Fig. 8. Effect of cycloheximide on secretion of triacylglycerol

Hepatocytes were incubated in DME medium supplemented with oleic acid (1.0 mM), BSA (0.13 mM), glycerol (5 μ Ci/ml, 25 μ M), without (○) or with (●) cycloheximide (20 μ M) for the indicated time periods.

When EGTA was added secretion of VLDL from the hepatocytes was markedly inhibited, probably because EGTA removes calcium from the cells. This is in accordance with previous findings demonstrating that EDTA inhibits secretion of triacylglycerol and cholesterol ester from suspended hepatocytes (Mathe *et al.*, 1984; Sundler *et al.*, 1973).

Several inorganic cations are reported to interfere with calcium metabolism at concentrations below 10 mM (Hagaiwara & Byerly, 1981). The relative inhibitory potency of the individual cations tested on VLDL secretion is in accordance with those reported for inhibition of calcium currents in nerve cells (Baker *et al.*, 1973; Hagaiwara & Byerly, 1981; Kostyuk, 1981) and inhibition of hormone release from adenohypophyseal secretory granules (Lorenson *et al.*, 1983). For instance, in smooth muscle, the relative efficacy to block calcium currents is lanthanum > nickel > cobalt > manganese (Hagaiwara & Byerly, 1981). Barium, which may replace calcium in nerve cells (Hagaiwara & Byerly, 1981; Kostyuk, 1981), did not affect secretion of VLDL. The concentration of cobalt necessary to decrease VLDL secretion is in the same range as that reported to affect insulin release, calcitonin secretion and calcium uptake in synaptosomes (Kotsias *et al.*, 1986; Maxwell & Sietz, 1983; Meyer & Cooper, 1983; Pento, 1980; Wollheim & Janjic, 1984). Cobalt may bind to voltage-dependent and to receptor-operated calcium channels as well as to intracellular calcium-binding sites (Thaw *et al.*, 1984; Wollheim & Janjic, 1984). The affinity of cobalt to these binding sites is very high, and this is probably the reason why the inhibitory effect is irreversible, even in the presence of calcium. Although the effect of cobalt was irreversible, the viability tests indicate that cobalt, as well as the other calcium-antagonists tested, did not injure the cells.

Optimal and reversible inhibition of triacylglycerol secretion was achieved at 100–200 μ M-verapamil. Although effects on calcium metabolism in this concentration range are reported (Botelho & Dartt, 1980; Malaisse, 1984), concentrations of verapamil below 10 μ M are normally used to inhibit calcium-dependent processes (Hagaiwara & Byerly, 1981; Russell & Thorn, 1974). Our observations therefore suggest that calcium influx through voltage-dependent calcium channels (inhibited by verapamil) is not of major importance for VLDL secretion. Rather, we believe that verapamil may inhibit VLDL secretion mainly by another mechanism. The drug is a weak base which may increase pH in acidic compartments of the secretory pathway and thereby inhibit secretion (Akiyama *et al.*, 1985; Anderson & Pathak, 1985; Drevon *et al.*, 1984, 1986; Nossen *et al.*, 1984; Rustan *et al.*, 1985).

Calcium may also be important in functions involving microtubules, microfilaments, enzyme activities, receptor–ligand interaction and fusion of transport vesicles with different cell membranes (Goldstein *et al.*, 1977; Malaisse, 1984; Maynard & Baenziger, 1982; Rasmussen, 1986; Williamson *et al.*, 1981). All these are related to VLDL secretion. It is possible that ‘intracellular receptors’ might be involved in secretion of VLDL in the same way as suggested for transport of proteins from endoplasmic reticulum to the Golgi complex (Lodish *et al.*, 1983). Binding of lipoproteins to these hypothetical receptors might be calcium-dependent, as shown for binding of several ligands (e.g. low-density lipoproteins and asialoglycoproteins) to their extracellular receptors (Goldstein *et al.*, 1977; Maynard & Baenziger, 1982).

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