# Prostaglandins suppress VLDL secretion in primary rat hepatocyte cultures: relationships to hepatic calcium metabolism

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**Abstract** In primary cultures of rat hepatocytes, prostaglandin $E_2$ and prostaglandin $D_2$ ($PGE_2$ and $PGD_2$) inhibited the secretion of very low density lipoprotein (VLDL)-associated apoB, triacylglycerol, and cholesterol. These effects were concentration-dependent and remained apparent for at least 3 days of culture without an effect on the apoB/triacylglycerol ratio of the secreted VLDL. Prostaglandins had no effect on the overall synthesis of triacylglycerol but triacylglycerol accumulated within the cells, without intracellular accumulation of apoB. $PGE_2$, when added to the medium together with glucagon, increased the inhibition of VLDL secretion, compared to that observed with glucagon alone. However, $PGE_2$ did not increase the stimulatory effect of glucagon on ketogenesis. Unlike glucagon, the prostaglandins did not inhibit fatty acid synthesis nor did they stimulate ketogenesis or production of cAMP. Thus, of all the parameters of hepatic lipid metabolism studied, $PGE_2$ and $PGD_2$ selectively affected VLDL. Selective inhibition of VLDL secretion was also observed with the calcium antagonist verapamil. The divalent cation ionophore A23187 also inhibited VLDL release but, in contrast, also inhibited fatty acid and cholesterol synthesis. The results suggest that VLDL secretion is modulated at some optimal cell calcium concentration that may be mediated selectively by agents such as prostaglandins.—**Björnsson, Ó. G., J. D. Sparks, C. E. Sparks, and G. F. Gibbons.** Prostaglandins suppress VLDL secretion in primary rat hepatocyte cultures: relationships to hepatic calcium metabolism. *J. Lipid Res.* 1992. **33:** 1017–1027.

**Supplementary key words** apolipoprotein B • triacylglycerol • cholesterol • verapamil • A23187 • extracellular fatty acids • lipid metabolism • cyclic AMP

Prostaglandins produced by Kupffer cells (1, 2) and hepatic sinusoidal endothelial cells (2) may regulate glucose metabolism and lipid oxidation by parenchymal liver cells (hepatocytes) (3–11), and it has therefore been suggested that prostaglandins have an important role in cellular communication within the liver (4, 7–10). Fatty acid synthesis, esterification and secretion as VLDL are important determinants of hepatic lipid balance and plasma lipid concentration (12). Little, however, is known of the possible role of prostaglandins in these areas of lipid metabolism. $PGD_2$ and $PGE_2$ are two of the main prostaglandins produced by the liver (1, 2), and we have studied, in isolated rat hepatocytes, the effects of these prostaglandins on hepatic synthesis and oxidation of fatty acids, synthesis of cholesterol, fatty acid esterification, and the secretion of VLDL apoB, triacylglycerol, and cholesterol. Also, the effects of prostaglandins on hepatic glycogenolysis have been proposed to be mediated via changes in concentration of cytosolic-free calcium (4) and/or cellular concentration of cAMP (3, 5). To explore the mechanism of action of prostaglandins in the liver, we have compared their effects with agents that affect intracellular levels of cAMP and $Ca^{2+}$ in rat hepatocytes.

## EXPERIMENTAL PROCEDURES

### Preparation of hepatocyte cultures

Hepatocytes from male Wistar rats (200–250 g body weight), fed ad libitum, were prepared under sterile conditions as described previously (13). After suspension ($0.7–0.9 \times 10^6$ cells/ml) in Waymouth's medium (MB 752/1) containing added amino acids, penicillin, streptomycin, and calf serum (13), the cells were plated into culture dishes (3.0 ml per dish). Four to 6 h later, when most of the cells had attached, the medium was removed and the cell monolayer was washed with phosphate-buffered saline (PBS). Serum-free Waymouth's Medium

Abbreviations: VLDL, very low density lipoprotein; cAMP, cyclic AMP; apoB, apolipoprotein B; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; $[Ca^{2+}]_i$, concentration of cytosolic free calcium; ER, endoplasmic reticulum; rER, rough endoplasmic reticulum; $PGE_2$, prostaglandin $E_2$; $PGD_2$, prostaglandin $D_2$; 16,16-dimethyl-$PGE_2$, 16,16-dimethyl-prostaglandin $E_2$.

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(3 ml) containing amino acids, antibiotics, dexamethasone (1 $\mu$M), lactate (10 mM), pyruvate (1 mM), and oleate (0.75 mM) was then added (referred to as the supplemented medium). Cells were cultured for periods of up to 72 h. In experiments where cells were cultured for more than 24 h, the culture medium was replaced by fresh medium every 24 h. Additions of prostaglandins, glucagon, or other compounds to be tested were made for the final 24-h period only (or shorter periods, if so stated; see legends to individual tables and figures).

### Harvesting of cells and isolation of VLDL

At the end of the culture period the culture medium was removed from the cells and the VLDL fraction was isolated as described previously (13, 14). An aliquot of 0.3 ml of the VLDL fraction was added to 0.3 ml of PBS containing 1% (w/v) of bovine serum albumin for analysis of apoB, and the samples were stored at $-20°$ to $-80°C$ until analyzed. Aliquots (0.5 ml) of the VLDL infranatant (d > 1.006 g/ml) were also stored at $-20°$ to $-80°C$ before analysis of apoB. Ice-cold PBS was added to the cell monolayer and the cells were removed from the dishes as described (13, 14).

### Measurement of cellular and VLDL triacylglycerol and cholesterol in cultured rat hepatocytes

Total lipids were extracted from the cellular pellet and the VLDL fraction by the method of Folch, Lees, and Sloane Stanley (15). The lipid residue was dissolved in 0.4 ml of ethanol, and the mass of triacylglycerol and cholesterol (nonesterified plus esterified) was measured using kits obtained from Boehringer-Mannheim (Triglycerides GPO-PAP and Cholesterol C-System, respectively). Manipulative losses of triacylglycerol were accounted for by addition of glycerol [$^{14}$C]trioleoylglycerol as an internal standard. In some experiments [$^{3}$H]oleate instead of nonradioactive oleate was added to the medium during the final 24 h of culture, and its contribution to the mass of cellular and VLDL triacylglycerol at the end of this period was measured from its specific radioactivity (see tables).

### Measurement of the rate of cholesterol and fatty acid synthesis in freshly prepared hepatocytes

Freshly prepared hepatocytes, isolated from male Wistar rats 2 h into the light phase of their cycle (16), were incubated at 37°C in a shaking water bath (70 cycles/min) for 2 h in a modified Krebs-Henseleit buffer (3.0 ml) containing calcium (2.5 mM), fatty acid-free bovine serum albumin (3.5% w/v), glucose (11.1 mM), amino acids (17), pyruvate (1 mM), [$^{14}$C]lactate (10 mM; 0.03 $\mu$Ci/$\mu$mol) and $^{3}H_2O$ (0.05 $\mu$Ci/$\mu$mol). Labeled fatty acids and cholesterol were isolated as described previously (18). The total mass of cholesterol and fatty acids synthesized was calculated from the incorporation of $^{3}H_2O$ (14, 16).

### Measurement of cellular apoB and apoB in VLDL and d > 1.006 g/ml infranatant

The apoB content of VLDL and of d > 1.006 g/ml infranatants was assayed by a competitive solid-phase monoclonal antibody radioimmunoassay using VLDL

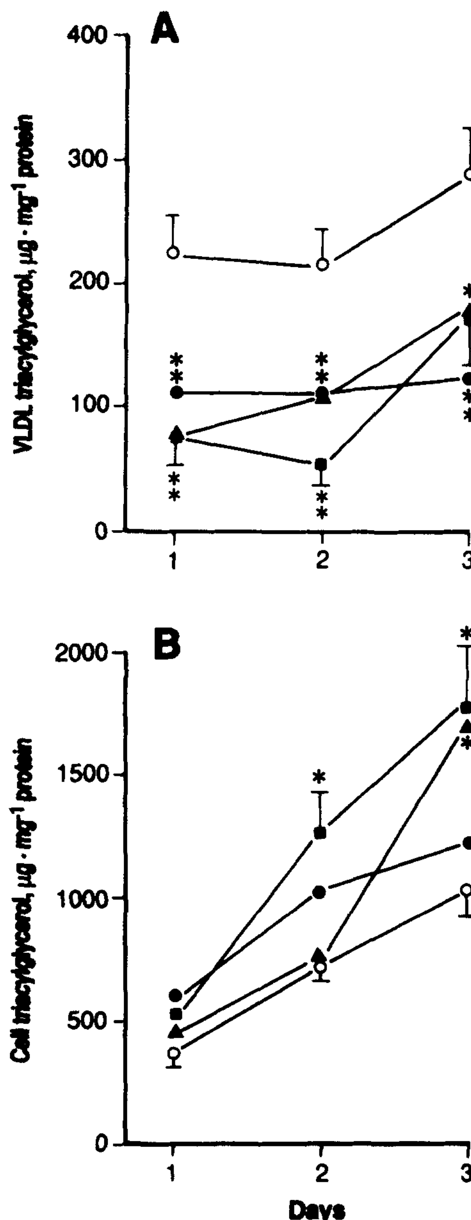


**Fig. 1.** Effects of prostaglandins on VLDL triacylglycerol secretion and on cellular levels of triacylglycerol in cultured rat hepatocytes. Hepatocytes were cultured for a total of 72 h (day 1, day 2, day 3, respectively), as described in Methods. Prostaglandins were present in the culture medium during the final 24 h of culture only. The data represent (A) VLDL triacylglycerol or (B) cellular levels of triacylglycerol, in the presence of prostaglandins during the final 24 h of culture: (● - ●) $PGE_2$ (5 × $10^{-5}$M); (▲ - ▲) $PGD_2$ (5 × $10^{-5}$M); (■ - ■) 16,16-dimethyl-$PGE_2$ (5 × $10^{-6}$M); (○ - ○) medium alone. The results are expressed as the means ± SEM of between five and eight independent hepatocyte preparations. $^{**}P \leq 0.01$; $^{*}P \leq 0.05$. (Unpaired "$t$" test).



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apoB as a standard (19). Control experiments demonstrated that the immunoreactivity of the apoB epitope recognized by this monoclonal antibody was not adversely affected by freezing or storage at −20°C or −80°C of VLDL samples after dilution in 1% bovine serum albumin. Addition of albumin to infranatants was not required because infranatants already contained sufficient albumin to stabilize the epitope upon freezing. Cellular apoB was measured in cells from dishes cultured under identical conditions to those used for assay of cellular lipids. In this case the cells were solubilized in barbital buffer containing Triton X-100 as described previously (20).

### Other methods

Cellular proteins were measured by a modified method of Lowry et al. (21). Oleate bound to bovine serum albumin (essentially fatty acid-free) was prepared by the method of van Harken, Dixon, and Heimberg (22). D-β-Hydroxybutyrate and acetoacetate were measured in the d > 1.006 g/ml infranatant by the method of Williamson, Mellanby, and Krebs (23). The results were expressed as the sum of these ketone bodies in the culture medium (μmol/ml). Cellular cAMP was measured by a radioimmunoassay using a kit purchased from Amersham International (U.K.) (cAMP [125] assay with Amerlex-M magnetic separation, product code RPA.509).

### Materials

The following compounds were purchased from Sigma Chemical Co. Ltd., Dorset, U.K.: glucagon (G-4250), $PGE_2$ (P-5640), $PGD_2$ (P-7025), and verapamil (V-4629). The divalent cation ionophore A23187 (Calcimycin) and 16,16-dimethyl-$PGE_2$ were bought from Novabiochem, Nottingham, U.K. Stock solutions of glucagon were prepared in sterile PBS. Stock solutions of all other compounds were prepared in pure DMSO (final concentration of DMSO not exceeding 0.33%, v/v). Controls had an equivalent amount of DMSO added to the culture dishes.

### Statistics

All values are presented as the means ± SEM of the number of independent experiments shown in parentheses (n) as stated in the legend of each table. Significant differences were assessed on the basis of paired or unpaired Student's $t$-test.

## RESULTS

### Prostaglandins ($PGE_2$, $PGD_2$, 16,16-dimethyl-$PGE_2$)

$PGE_2$ or $PGD_2$ suppressed VLDL secretion of apoB, triacylglycerol, and cholesterol in rat hepatocytes (**Fig. 1A** and **Table 1**). Suppression of VLDL secretion of triacyl-

TABLE 1. Secretion of VLDL cholesterol and apoB by cultured rat hepatocytes: effects of 24 h exposure to prostaglandins during 3 successive days of culture

| Incubation Conditions | Culture Period | VLDL Cholesterol | VLDL ApoB | VLDL ApoB/Triacylglycerol |
|---|---|---|---|---|
| | | *μg/mg/24 h* | *ng/mg/24 h* | × *1000* |
| Control | 4–28 h (day 1) | 10.5 ± 2.1 | 1532 ± 577 | 7.2 ± 1.5 |
| $PGE_2$, 5 × $10^{-5}$M | | 5.2 ± 0.6[a] | 546 ± 355[a] | 5.1 ± 1.2 |
| 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | | 3.5 ± 0.3[a] | 591 ± 196[a] | 9.8 ± 0.8 |
| $PGD_2$, 5 × $10^{-5}$M | | 5.4 ± 2.3[a] | 504 ± 366[a] | 7.0 ± 1.4 |
| Control | 24–48 h (day 2) | 8.5 ± 2.0 | 1196 ± 290 | 4.1 ± 1.1 |
| $PGE_2$, 5 × $10^{-5}$M | | 4.0 ± 0.7[a] | 378 ± 143[a] | 3.6 ± 0.9 |
| 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | | 4.6 ± 0.8[a] | 372 ± 132[a] | 4.4 ± 0.8 |
| $PGD_2$, 5 × $10^{-5}$M | | 5.6 ± 1.5[b] | 698 ± 166[a] | 7.5 ± 2.5 |
| Control | 48–72 h (day 3) | 8.9 ± 2.5 | 1582 ± 492 | 3.7 ± 0.7 |
| $PGE_2$, 5 × $10^{-5}$M | | 3.1 ± 0.7[c] | 473 ± 200[a] | 3.9 ± 0.9 |
| 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | | 2.9 ± 0.7[a] | 540 ± 283[a] | 5.4 ± 1.3 |

Hepatocytes were cultured for a total of 72 h in the supplemented medium. For hepatocytes cultured longer than 24 h (i.e., day 2, day 3), the medium was changed at intervals of 24 h. Prostaglandins were present in the medium during the final 24 h of culture only. At the end of the final 24 h culture period the medium was removed and the VLDL and the VLDL infranatant (d > 1.006 g/ml) fractions were obtained. Rates of secretion of VLDL cholesterol (μg/mg protein/24 h) and of apoB (ng/mg protein/24 h) were determined as described in Methods. Results are expressed as the mean ± SEM of four to six independent experiments.

[a] Significantly different from their corresponding control means at a probability of at least $P < 0.05$.

[b] Different from the corresponding control mean at $P = 0.05$.

[c] Significantly different from the corresponding control means at a probability of at least $P < 0.01$.



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glycerol by $PGE_2$ was observed after the first 24 h of culture, and was maintained when the prostaglandins were added for the final 24 h of 48 h or 72 h cultures (day 2 or day 3, respectively) (Fig. 1A). In other words, the cells were responsive to the inhibitory effects of prostaglandins on the secretion of VLDL for at least 3 days in culture. The suppression of VLDL secretion by $PGE_2$ was dependent on its concentration, and was observed at concentrations as low as $5 \times 10^{-7}$M (**Fig. 2**). The potency of $PGE_2$ and $PGD_2$ in suppressing VLDL secretion was similar, but the stable prostaglandin $E_2$ analogue 16,16-dimethyl-$PGE_2$ was at least one order of magnitude more potent than $PGE_2$ (Fig. 1A). The suppression of VLDL apoB secretion observed in cells incubated with prostaglandins was not likely to be due to redistribution of secreted apoB between VLDL and lipoproteins of higher density (14), since the prostaglandins suppressed the levels of apoB detected in the d > 1.006 g/ml infranatant to a similar extent (data not shown). $PGE_2$ did not have a selective effect on the inhibition of VLDL apoB secretion compared to VLDL triacylglycerol, as shown by the similarity in the apoB/triacylglycerol ratio during each of the 3 days of culture (Table 1). However, the apoB/triacylglycerol ratio tended to decrease with time of culture.

The suppression of VLDL secretion by 16,16-dimethyl-$PGE_2$ and, to a lesser extent, that by $PGD_2$ was accompanied by accumulation of triacylglycerol within the cells (Fig. 1B). During incubation with 16,16-dimethyl-$PGE_2$ ($5 \times 10^{-6}$M), there was also a cellular accumulation of cholesterol newly synthesized from exogenous [$^3$H]oleate ($56.5 \pm 14.6$ and $113.6 \pm 28.3$ nmol oleate incorporated/mg protein per 24 h, during day 1 and day 2, respectively, vs. control, $30.4 \pm 4.9$ and $68.1 \pm 18.6$ nmol/mg protein per 24 h (n = 5-6, $P < 0.05$). However, no net accumulation of cellular apoB was observed in cells during incubation with prostaglandins (16,16-dimethyl-$PGE_2$, ($5 \times 10^{-6}$M), $407 \pm 60$ ng/mg protein; $PGD_2$, ($5 \times 10^{-5}$M), $328 \pm 87$ ng/mg protein, vs. control, $336 \pm 44$ ng/mg protein, $P > 0.05$, n = 4-5).

Prostaglandins had no effect on the overall rate of triacylglycerol synthesis from exogenous fatty acids (**Table 2**). This is represented by the sum of cellular and VLDL triacylglycerol labeled from exogenously added [$^3$H]oleate (Table 2). The effects of prostaglandins on the other major contributors to hepatic fatty acid metabolism (de novo synthesis of fatty acids and fatty acid oxidation) were also determined. Fatty acid synthesis was assessed by measuring the incorporation of $^3H_2O$ and [$^{14}$C]lactate into newly synthesized fatty acids. $PGE_2$, $PGD_2$, or 16,16-dimethyl-$PGE_2$ had little effect on the overall flux of carbon into fatty acids (represented by $^3H_2O$ incorporation) (**Table 3**). However, the specific contribution of [$^{14}$C]lactate to overall fatty acid synthesis declined somewhat in the presence of $PGD_2$ and 16,16-dimethyl-$PGE_2$ (Table 3). As regards cholesterol synthesis, only 16,16-dimethyl-$PGE_2$ had a significant inhibitory effect on $^3H_2O$ or [$^{14}$C]lactate incorporation (Table 3). In general, the effects of the prostaglandins on the synthesis of fatty acids were much less pronounced than those on the secretion of VLDL. The disposal of hepatic fatty acids by oxidation was assessed by measuring the rates of ketogenesis. None of the

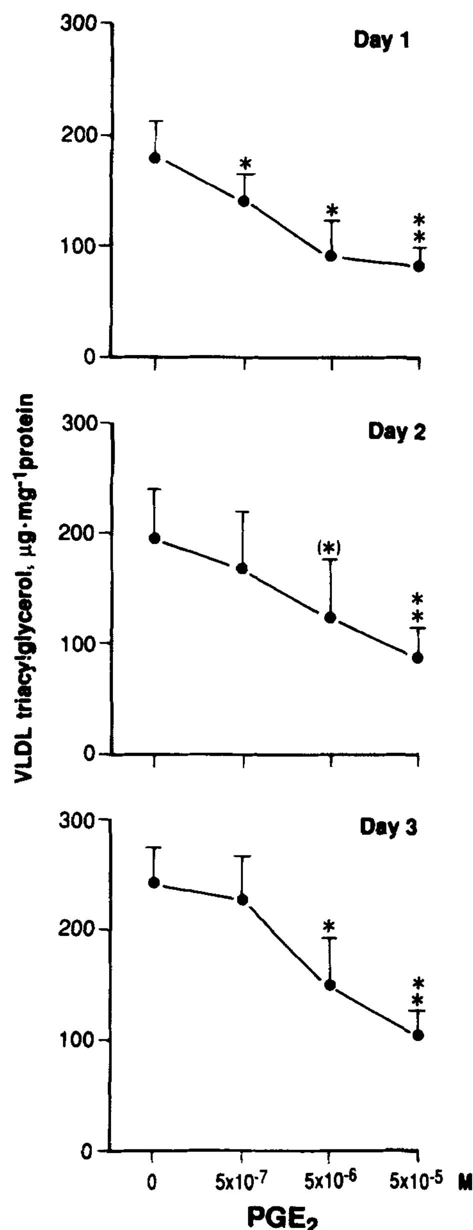


**Fig. 2.** $PGE_2$-induced suppression of VLDL triacylglycerol secretion in rat hepatocytes in culture; effect of time and concentration. Hepatocytes were cultured for a total of 72 h (day 1, day 2, day 3, respectively) according to described procedures (see Methods). $PGE_2$ of varying concentrations ($5 \times 10^{-7}$M, $5 \times 10^{-6}$M, $5 \times 10^{-5}$M) was present in the culture medium during the final 24 h of culture only. The results are expressed as the means ± SEM of between three and five independent hepatocyte preparations. **$P \leq 0.02$; *$P \leq 0.05$; (*)$P \sim 0.06$. (Unpaired "*t*" test).



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TABLE 2. Effects of prostaglandins on the synthesis of triacylglycerol from oleate by cultured rat hepatocytes

| Culture Period | Incubation Conditions | Cells | Total (Cells + VLDL) |
|---|---|---|---|
| *h* | | | |
| 4-28 | Control | 0.79 ± 0.18 | 1.21 ± 0.17 |
| | $PGE_2$, 5 × $10^{-5}$M | 1.51 ± 0.55 | 1.80 ± 0.53 |
| | $PGD_2$, 5 × $10^{-5}$M | 0.94 ± 0.24 | 1.04 ± 0.24 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 0.93 ± 0.19 | 1.07 ± 0.21 |
| 24-48 | Control | 1.43 ± 0.20 | 1.83 ± 0.20 |
| | $PGE_2$, 5 × $10^{-5}$M | 1.65 ± 0.27 | 1.85 ± 0.27 |
| | $PGD_2$, 5 × $10^{-5}$M | 1.17 ± 0.25 | 1.36 ± 0.22 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 2.01 ± 0.49 | 2.11 ± 0.48 |
| 48-72 | Control | 1.43 ± 0.72 | 1.88 ± 0.37 |
| | $PGE_2$, 5 × $10^{-5}$M | 1.74 ± 0.45 | 2.14 ± 0.49 |
| | $PGD_2$, 5 × $10^{-5}$M | 2.02 ± 0.56 | 2.90 ± 0.44 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 1.89 ± 0.56 | 2.72 ± 0.54 |

Rat hepatocytes were cultured for a total of 72 h as described (see Methods). [$^3$H]oleate (0.75 mM; 9.78 × $10^5$ dpm/µmol) and the prostaglandins tested were present during the final 24 h of each culture period only. At the end of that 24 h period the cellular and VLDL triacylglycerol were isolated and their radioactivity was determined. In each experiment the sum of the label in the cellular and VLDL triacylglycerol was taken to represent total triacylglycerol synthesis. The above values are µmol triacylglycerol synthesized per mg cell protein. Results are expressed as means ± SEM of five to eight independent experiments.

prostaglandins studied had any significant effect on ketogenesis in hepatocytes cultured for up to a total of 72 h (data not shown). Thus, of all the metabolic pathways that contribute to hepatic fatty acid and triacylglycerol balance, only one, the secretion of VLDL, was affected by the prostaglandins.

In experiments where [$^3$H]oleate instead of nonradioactive oleate was added to the supplemented medium during the final 24 h of culture, the specific radioactivity of intracellular and secreted triacylglycerol were similar when [$^3$H]oleate was added either during the first or the second 24 h of culture (day 1 and day 2, respectively), and this occurred irrespective of the presence or absence of prostaglandins in the medium (**Table 4**). This pattern suggests complete attainment of equilibrium between intracellular and secreted triacylglycerol after 24 h. This is in contrast to previous shorter-term studies (24) in which the difference in specific radioactivity between these pools indicated that equilibrium had not yet been reached. When [$^3$H]oleate was added during the final 24 h of a 72-h culture period (day 3), the specific activity both in the intracellular and the secreted triacylglycerol was less than in the corresponding experiments carried out on day 1, indicating a decreased relative contribution to both of

TABLE 3. Synthesis of fatty acids and cholesterol in freshly prepared rat hepatocytes: effects of prostaglandins, verapamil, and A23187

| | Fatty Acid Synthesis, % of Control | | Cholesterol Synthesis, % of Control | |
|---|---|---|---|---|
| | $^3H_2O$ Incorp. | [$^{14}$C]Lactate Incorp. | $^3H_2O$ Incorp. | [$^{14}$C]Lactate Incorp. |
| Control | 100 | 100 | 100 | 100 |
| $PGE_2$, 5 × $10^{-5}$M | 92.4 ± 4.1 | 90.9 ± 5.3 | 100.5 ± 3.7 | 97.2 ± 6.0 |
| $PGD_2$, 5 × $10^{-5}$M | 85.6 ± 6.9 | 73.6 ± 8.5[a] | 106.0 ± 13.4 | 90.8 ± 13.8 |
| 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 83.2 ± 10.5 | 60.3 ± 4.9[a] | 66.8 ± 10.0[b] | 49.0 ± 3.8[b] |
| Verapamil, $10^{-5}$M | 104.7 ± 4.9 | 105.7 ± 6.3 | 109.7 ± 15.0 | 115.3 ± 12.9 |
| A23187, $10^{-5}$M | 25.0 ± 6.2[b] | 20.8 ± 6.9[b] | 37.9 ± 8.7[b] | 31.1 ± 7.3 [b] |

Freshly prepared hepatocytes were incubated for 2 h in the absence (control) or presence of prostaglandins, verapamil, or A23187. Data represent de novo fatty acid synthesis or de novo cholesterol synthesis during the 2 h of incubation, estimated as incorporation of $^3H_2O$ or [$^{14}$C]lactate (see Methods). Data are expressed as percentage of control; mean ± SEM of four to seven independent experiments.

[a] $P \leq 0.05$.

[b] $P \leq 0.01$.

Control values, fatty acid synthesis: 10.1 ± 0.9 nmol $^3H_2O$/mg protein, 26.3 ± 3.0 nmol [$^{14}$C]lactate/mg protein.

Control values, cholesterol synthesis: 1.1 ± 0.2 nmol $^3H_2O$/mg protein, 3.0 ± 0.6 nmol [$^{14}$C]lactate/mg protein.



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TABLE 4. Effects of prostaglandins on the contribution of exogenous oleate to VLDL- and intracellular triacylglycerol in cultured rat hepatocytes

| Culture Period during Which [$^3$H]Oleate Was Present | Incubation Conditions | % of Triacylglycerol due to [$^3$H]Oleate | |
|---|---|---|---|
| | | Cell | VLDL |
| *h* | | | |
| 4-28 (Day 1) | Control | 60.9 ± 6.3 | 59.7 ± 5.3 |
| | $PGE_2$, 5 × $10^{-5}$M | 58.8 ± 13.1 | 51.2 ± 6.7 |
| | $PGD_2$, 5 × $10^{-5}$M | 62.3 ± 14.6 | 44.8 ± 10.0 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 54.8 ± 7.6 | 45.4 ± 14.1 |
| 24-48 (Day 2) | Control | 57.6 ± 6.4 | 57.4 ± 6.8 |
| | $PGE_2$, 5 × $10^{-5}$M | 51.5 ± 10.1 | 61.8 ± 6.1 |
| | $PGD_2$, 5 × $10^{-5}$M | 49.6 ± 9.6 | 61.9 ± 8.8 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 49.6 ± 10.3 | 35.3 ± 10.4 |
| 48-72 (Day 3) | Control | 41.0 ± 5.3[a] | 46.8 ± 3.8 |
| | $PGE_2$, 5 × $10^{-5}$M | 49.4 ± 13.2 | 45.1 ± 4.3 |
| | $PGD_2$, 5 × $10^{-5}$M | 32.3 ± 5.1[a,b] | 46.6 ± 8.0 |
| | 16,16-Dimethyl-$PGE_2$, 5 × $10^{-6}$M | 29.2 ± 4.4[a,b] | 40.2 ± 4.1 |

Rat hepatocytes were cultured for various periods up to a total of 72 h in the supplemented medium. During the periods shown, non-radioactive oleate was replaced by [$^3$H]oleate (0.75 mM; 9.78 × $10^5$ dpm/$\mu$mol) and the prostaglandins ($PGE_2$, $PGD_2$, 16,16-dimethyl-$PGE_2$) were either absent (control) or present in the medium. At the end of each period the cellular and VLDL triacylglycerol was extracted. Aliquots of each fraction were used for determination of total mass and $^3$H radioactivity. The mass of oleate that had entered each pool was calculated on the basis of its specific radioactivity. This quantity was used to calculate its contribution (%) to the total mass of each pool. Results are presented as the means ± SEM of between five and nine independent experiments.

[a]Significantly different ($P \leq 0.01$) from the control values observed in the cell fraction on day 1.

[b]Significantly different ($P \leq 0.05$) from the corresponding values observed in the VLDL fraction on day 3.

these pools. The specific activity was particularly low in the intracellular triacylglycerol during day 3 of culture in cells incubated with either $PGD_2$ or the stable analogue 16,16-dimethyl-$PGE_2$. In these experiments the specific radioactivity in the cellular triacylglycerol was lower than that in the corresponding VLDL fraction secreted during the same day (day 3) (Table 4).

**Effects of $PGE_2$ in the presence of glucagon**

Certain prostaglandins have been shown to attenuate the effects of glucagon on glycogenolysis (6, 10) and fatty acid oxidation (5) in hepatocytes by decreasing the glucagon-mediated rise in intracellular cAMP. Glucagon also suppresses the secretion of hepatic VLDL (14, 25), and it was therefore of interest to determine whether this inhibitory effect was attenuated by prostaglandins. This was not the case. In fact, $PGE_2$, when present simultaneously with glucagon, suppressed the secretion of VLDL lipids to a greater extent than that observed with glucagon alone (**Table 5**). Despite this, the stable prostaglandin analogue 16,16-dimethyl-$PGE_2$ (5 × $10^{-6}$M) suppressed the glucagon ($10^{-7}$M)-mediated increase in intracellular

TABLE 5. Effects of prostaglandins on the secretion of VLDL in the presence of glucagon

| Addition to Medium | Secretion of VLDL Triacylglycerol | |
|---|---|---|
| | Total | Newly Synthesized |
| | *μg/mg protein* | *μmol oleate/mg protein* |
| None | 227 ± 33 (100%) | 0.42 ± 0.08 (100%) |
| Glucagon ($10^{-7}$M) | 141 ± 35 (55.5 ± 7.1%)[a] | 0.25 ± 0.07 (56.3 ± 8.5%)[a] |
| $PGE_2$ (5 × $10^{-5}$M) | 110 ± 31 (50.7 ± 6.1%)[a] | 0.16 ± 0.02 (49.6 ± 6.0%)[a] |
| Glucagon ($10^{-7}$M) + $PGE_2$ (5 × $10^{-5}$M) | 80 ± 24 (34.9 ± 4.4%)[a,b] | 0.10 ± 0.02 (32.6 ± 4.6%)[a,b] |

Hepatocytes were cultured as described in the Methods section. Glucagon and $PGE_2$ were present between 4 h and 28 h after plating of the cells (day 1) in the supplemented culture medium in which non-radioactive oleate was replaced by [$^3$H]oleate of the same initial concentration. The secretion of total and labeled VLDL triacylglycerol into the medium was determined. Results are expressed as the mean ± SEM of between six and ten independent experiments.

[a]Significantly different from the control values ($P < 0.01$).

[b]Significantly different from values observed in the presence of glucagon alone ($P < 0.05$).



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cAMP concentration (139 ± 28 pmol/mg protein (glucagon) vs. 31 ± 11 pmol/mg protein (16,16-dimethyl-$PGE_2$ + glucagon), $P < 0.05$, n = 6), as shown previously for $PGE_2$ (26). This occurred 10 min after addition of the compounds, the time at which the glucagon-mediated increase in cAMP was maximal (14). At this time point, $PGE_2$ alone (5 × $10^{-5}$M) had little or no effect on the basal levels of cAMP (12 ± 2 pmol/mg protein vs. 8 ± 3 pmol/mg protein control). 16,16-Dimethyl-$PGE_2$ also decreased the glucagon-mediated stimulation of ketogenesis to 62 ± 14% of that observed in the presence of glucagon alone ($P < 0.05$). It appeared, therefore, that the inhibitory effects of prostaglandins on the secretion of VLDL were not mediated by changes in cAMP. Another possibility, suggested by the influence of $PGE_2$ on the metabolic effects of the $\alpha_1$-adrenergic agonist phenylephrine (3) and of the divalent cation ionophore A23187 (3), is that the inhibition of VLDL secretion may be signalled by changes in concentration of cytosolic free $Ca^{2+}$. To investigate this possibility, the metabolic effects of the prostaglandins were compared with those of the ionophore A23187 and with those of the $Ca^{2+}$ antagonist, verapamil.

### Compounds that affect cellular calcium homeostasis (verapamil, A23187)

Compounds that either suppress (verapamil) or increase (A23187) the concentration of cytosolic free $Ca^{2+}$, $[Ca^{2+}]_i$, suppressed the secretion of VLDL apoB and triacylglycerol in a dose-dependent manner (**Fig. 3**). A similar inhibition of VLDL-associated cholesterol was also observed (results not shown). The inhibition of VLDL secretion was paralleled by accumulation of triacylglycerol within the cells (Fig. 3B, D) (estimated as total mass). A similar cellular accumulation of newly synthesized triacylglycerol or newly synthesized cholesterol (estimated as incorporation of $^3H$ from added [$^3H$]oleate) was also observed (results not shown). In common with the prostaglandins, verapamil or A23187 had no effect on the overall rate of synthesis of triacylglycerol from exogenous

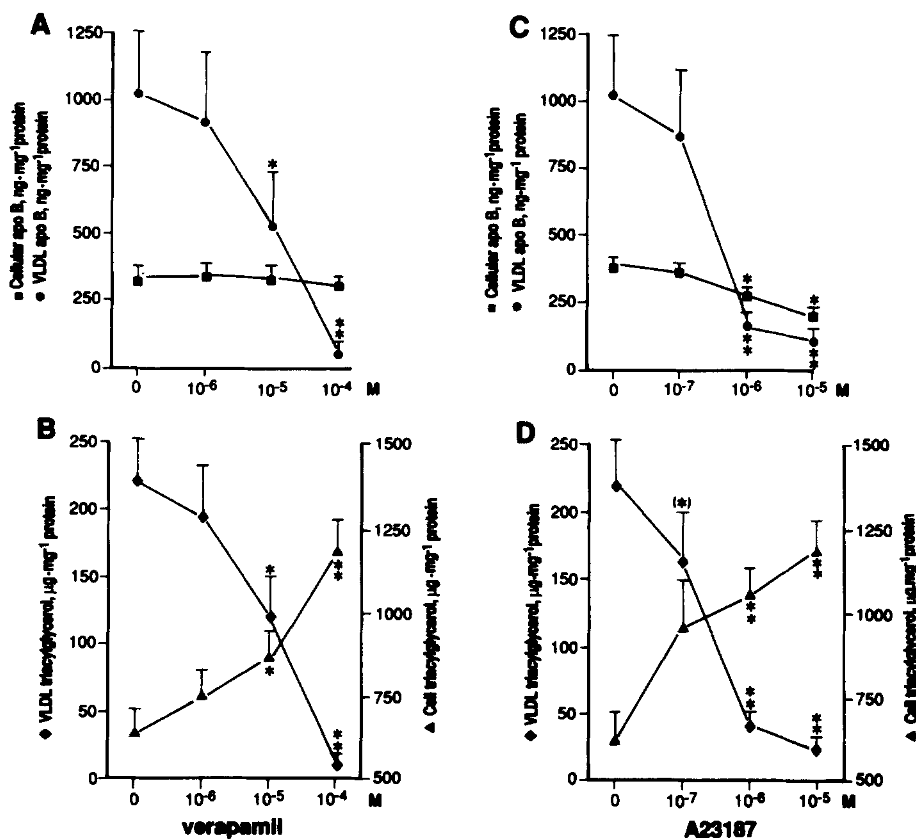


**Fig. 3.** VLDL secretion of triacylglycerol and apoB and cellular levels of triacylglycerol and apoB in cultured rat hepatocytes incubated with compounds that affect cellular calcium homeostasis. Rat hepatocytes were cultured for a total of 48 h in the supplemented medium. During the final 24 h of culture, verapamil (A, B) or A23187 (C, D) was either present or absent in the medium: (● - ●) VLDL apoB; (■ - ■) cellular apoB; (◆ - ◆) VLDL triacylglycerol; (▲ - ▲) cellular triacylglycerol. The results are expressed as means ± SEM of between six and nine independent experiments. The concentration of calcium in the Waymouth's culture medium was 0.8 mM. **$P \leq 0.01$; *$P \leq 0.05$; (*)P = 0.06. (Unpaired "*t*" test).



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TABLE 6. Effects of verapamil or A23187 on the synthesis of triacylglycerol from oleate by cultured rat hepatocytes

| Culture period | Incubation Conditions | Triacylglycerol Synthesized: Cells | Triacylglycerol Synthesized: Total (Cells + VLDL) |
|---|---|---|---|
| *h* | | *μmol oleate/mg cell protein/24 h* | |
| 24-48 | Control | 1.29 ± 0.21 | 1.73 ± 0.26 |
| | Verapamil, $10^{-4}$M | 1.80 ± 0.42 | 2.00 ± 0.46 |
| | Verapamil, $10^{-5}$M | 1.51 ± 0.32 | 1.74 ± 0.35 |
| | Verapamil, $10^{-6}$M | 1.26 ± 0.25 | 1.64 ± 0.34 |
| | A23187, $10^{-5}$M | 1.96 ± 0.27 | 1.97 ± 0.27 |
| | A23187, $10^{-6}$M | 1.91 ± 0.35 | 1.95 ± 0.36 |
| | A23187, $10^{-7}$M | 1.54 ± 0.35 | 1.88 ± 0.37 |

Hepatocytes were cultured for a total of 72 h as described in Methods. [$^3$H]oleate (0.75 mM; 9.78 × $10^5$ dpm/μmol) and the compounds tested were present during the final 24 h of cell culture only. Results are expressed as the mean ± SEM of six to eight independent experiments.

fatty acids (**Table 6**), as assessed by the sum of [$^3$H]oleate incorporated into cellular and VLDL triacylglycerol. Verapamil, at a concentration ($10^{-5}$M) that significantly suppressed the secretion of VLDL (Fig. 3), also had no effect on the rate of de novo fatty acid synthesis (Table 3). Even at a concentration of $10^{-4}$M, which almost completely blocked the secretion of apoB, cholesterol, and triacylglycerol, verapamil inhibited fatty acid synthesis by only 20% (results not shown). Similar effects were also observed on the rates of cholesterogenesis (Table 3). By contrast, the divalent cation ionophore A23187, at a concentration of $10^{-5}$M, suppressed fatty acid and cholesterol synthesis rates by 75% and 62%, respectively (Table 3). When [$^3$H]oleate was added during the final 24 h of a 48-h culture period (day 2), the specific activity of the VLDL triacylglycerol fraction was decreased in a dose-dependent manner in cells incubated with A23187 (**Table 7**), and the specific activity was lower than in the corresponding cellular fraction. Neither verapamil nor A23187 had any effect on ketogenesis, irrespective of the concentration used (**Fig. 4**). Verapamil ($10^{-4}$M) or A23187 ($10^{-5}$M) had no effect on cellular levels of cAMP (6.1 and 7.2 pmol/mg protein, respectively, vs. 7.3 pmol/mg protein, control (10 min), n = 3, $P > 0.05$).

## DISCUSSION

$PGE_2$ suppressed VLDL secretion of apoB, triacylglycerol, and cholesterol in primary cultures of rat hepatocytes. The inhibition of VLDL secretion occurred without a change in VLDL particle size as indicated by

TABLE 7. Effects of verapamil or A23187 on the contribution of exogenous oleate to VLDL- and intracellular triacylglycerol in cultured rat hepatocytes

| Culture Period during Which [$^3$H]Oleate Was Present | Incubation Conditions | % of Triacylglycerol due to [$^3$H]Oleate: Cell | % of Triacylglycerol due to [$^3$H]Oleate: VLDL |
|---|---|---|---|
| *h* | | | |
| 24-48 | Control | 49.5 ± 5.8 | 64.1 ± 9.6 |
| | Verapamil, $10^{-4}$M | 48.2 ± 7.2 | 51.7 ± 10.7 |
| | Verapamil, $10^{-5}$M | 50.3 ± 6.6 | 64.8 ± 10.9 |
| | Verapamil, $10^{-6}$M | 52.8 ± 9.0 | 64.1 ± 12.3 |
| | A23187, $10^{-5}$M | 49.8 ± 6.3[a] | 17.0 ± 9.6[b] |
| | A23187, $10^{-6}$M | 52.3 ± 7.5[a] | 32.3 ± 10.5[c] |
| | A23187, $10^{-7}$M | 50.1 ± 7.3 | 63.3 ± 10.3 |

Rat hepatocytes were cultured for 48 h as described in Methods. Verapamil ($10^{-4}$M-$10^{-6}$M) or A23187 ($10^{-5}$ M-$10^{-7}$M) was present in the medium during the final 24 h period of culture only. Results are expressed as the mean ± SEM of between six and eight independent experiments.
[a]Significantly different from the corresponding values observed in the VLDL fraction ($P \leq 0.05$).
[b]Significantly different from the corresponding control value for VLDL triacylglycerol ($P < 0.01$).
[c]Significantly different from the corresponding control value for VLDL triacylglycerol ($P < 0.05$).



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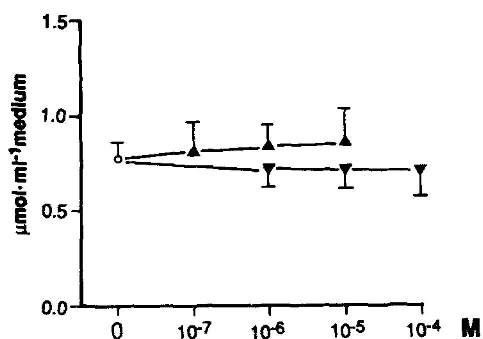


**Fig. 4.** Ketogenesis of rat hepatocytes cultured in the presence of increasing concentrations of verapamil or A23187. Hepatocytes were cultured for 48 h in the supplemented medium. Verapamil (▼ - ▼), or A23187 (▲ - ▲) was present in the culture medium during the final 24 h of culture only, (○) control. The results are expressed as the means ± SEM of six to eight independent experiments. For further details, see the legend to Fig. 3.

the similarity in the apoB/triacylglycerol ratio (27) in the presence and absence of $PGE_2$. Similar results were obtained from hepatocytes incubated for 24 h in a medium containing either $PGD_2$ or the stable prostaglandin analogue 16,16-dimethyl-$PGE_2$. The VLDL suppression was accompanied by accumulation of triacylglycerol and cholesterol within the cells. The suppression of VLDL secretion by 16,16-dimethyl-$PGE_2$ was at least one order of magnitude greater than the effects of $PGE_2$ or $PGD_2$. Rapid metabolism of prostaglandins by hepatocytes (28, 29) is a likely explanation for this, but it cannot be excluded that 16,16-dimethyl-$PGE_2$ may also be a more potent compound on a molar basis than the natural prostaglandins. Although the concentrations of the natural prostaglandins used were considerably higher than might be expected in peripheral blood, these higher concentrations were used to compensate for extensive biological degradation (28, 29). Moreover, prostaglandins produced by the liver may be present locally in far higher concentrations than those in peripheral blood. None of the prostaglandins tested changed the overall rate of triacylglycerol synthesis in the present studies, nor did they affect de novo synthesis of fatty acids or cholesterol. The prostaglandins were also without an effect on the basal rate of hepatic ketogenesis, which suggests that they did not affect the $\beta$-oxidation of fatty acids. Thus, unlike pancreatic hormones, which have regulatory effects across a wide spectrum of hepatic lipid metabolism (14, 25, 30), the prostaglandins seem to affect, specifically, the secretion of hepatic VLDL.

This selectivity shown by the prostaglandins was also seen with the calcium antagonist verapamil (31, 32). This compound, although inhibiting the secretion of VLDL triacylglycerol, apoB, and cholesterol at concentrations at least as low as 10 $\mu$M (Fig. 3), had little or no effect on the other parameters of lipid metabolism studied. However, in shorter-term studies than those reported here, Olubadewo, Cook, and Heimberg (33) showed that verapamil (50 $\mu$M) inhibited ketogenesis by 38%. The apparent discrepancy between this and the present results may reflect the large difference in the time periods over which these effects were studied. The effects of verapamil on triacylglycerol secretion support those of Nossen, Rustan, and Drevon (34) who observed, in short-term incubations of freshly prepared rat hepatocytes, that this $Ca^{2+}$ antagonist suppressed VLDL triacylglycerol secretion, but apparently without affecting triacylglycerol synthesis. Our results also support the findings of Kwong et al. (35) who found that the calcium antagonist diltiazem inhibited secretion of apoB by hepatocytes. The present results suggest a role for prostaglandins in suppressing the secretion of VLDL, possibly by inhibiting flux of $Ca^{2+}$. This view is supported by the observation that prostaglandins suppress the effects of $\alpha$-adrenergic agonists on glycogenolysis (3, 6, 10). The present results, however, cannot distinguish between effects on apoB at the transcriptional, translational, or post-translational levels.

The observation that the divalent cation ionophore A23187 suppressed VLDL secretion was therefore unexpected. A23187 is predominantly selective for divalent cations (36), and has been shown to raise $[Ca^{2+}]_i$ in rat hepatocytes. It also causes efflux of $Ca^{2+}$ in hepatocytes incubated in low $Ca^{2+}$ buffer (32, 37). A rise in $[Ca^{2+}]_i$ is associated with exocytosis in several cell types (38), and even though little is known about the role of $Ca^{2+}$ in the early stages of cellular protein synthesis and secretion, Lodish and Kong reported recently (39) that in cultured human hepatoma cells (HepG2 cells), A23187 or the $Ca^{2+}$ ionophore ionomycin blocked transport of several secretory proteins from the rough endoplasmic reticulum (rER). This inhibition was partly prevented by increasing the $Ca^{2+}$ concentration of the extracellular culture medium. Lodish and Kong (39) suggested that these observations could be explained if a high concentration of $Ca^{2+}$ in the ER lumen was required for correct protein folding, defects of which result in protein retention within the ER and degradation (40, 41). In this respect, the retention of apoB in the ER induced by Brefeldin A resulted in increased loss of intact apoB-100 (42). However, it has been shown recently that a decrease in the luminal $Ca^{2+}$ concentration of the ER enhances the release of certain secretory proteins by preventing their binding to an ER retention molecule (43). In the present studies no net increase in cellular apoB was observed after 24 h culture with verapamil or A23187, despite suppression of VLDL secretion by these compounds and net accumulation of triacylglycerol and cholesterol. Thus, verapamil and A23187 led to a decrease in the total amount of apoB produced by the cell (**Table 8**) and suggests that the intracellular triacylglycerol which accumulated under these conditions was not VLDL. The similar effects of verapamil and A23187 on apoB metabolism, despite their



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TABLE 8. Effect of verapamil and A23187 on the total amount of immunoreactive apoB

| Addition to Cells | Total ApoB |
|---|---|
| Experiment A | |
| None | 3109 ± 624 |
| A23187 ($10^{-5}$M) | 881 ± 206[a] |
| A23187 ($10^{-6}$M) | 1987 ± 502[a] |
| A23187 ($10^{-7}$M) | 3291 ± 985 |
| Experiment B | |
| None | 2901 ± 656 |
| Verapamil ($10^{-4}$M) | 485 ± 134[a] |
| Verapamil ($10^{-5}$M) | 2158 ± 783[b] |
| Verapamil ($10^{-6}$M) | 2908 ± 864 |

The amounts of apoB present in the cells (Fig. 4) were added to those present in the VLDL (Fig. 4) and d > 1.006 g/ml infranatant fractions. The values represent the mean ± SEM of between six and nine independent experiments.
[a]Significantly different from the corresponding controls ($P < 0.01$).
[b]Significantly different from the corresponding controls ($P < 0.05$).

different effects on $Ca^{2+}$ mobilization from the ER (31, 32, 37, 39), suggest that there is an optimal concentration of cell $Ca^{2+}$ that is required for efficient transport of apoB through the secretory apparatus.

In other respects, the effects of A23187 on hepatic lipid metabolism differed from those of verapamil. For instance, A23187 suppressed cholesterol and fatty acid synthesis, whereas verapamil did not (Table 3). This effect of A23187 may have resulted from decreased activities of key regulatory enzymes for these pathways as has already been shown for HMG-CoA reductase (44). A23187, in the presence of $Ca^{2+}$ has previously been shown to mimic the short-term effects of glucagon on lipid metabolism in hepatocytes (45). In particular, there was a decreased rate of triacylglycerol synthesis which contrasts with the present observations (Table 6). However, we (14) and others (46) have shown that longer-term exposure of hepatocytes to glucagon does not suppress triacylglycerol synthesis and this is consistent with the ineffectiveness of A23187 in this regard in the longer term (Table 6). Neither did A23187 stimulate ketogenesis (Fig. 4) as might have been expected from the short-term studies (45).

The present results suggest that prostaglandins of the D and E series are capable of regulating the secretion of hepatic VLDL in vitro without affecting other aspects of hepatocellular lipid metabolism. That this may be of physiological importance is suggested by the presence of receptors for $PGE_2$ and $PGD_2$ on rat hepatocyte plasma membranes (9, 47), and also by the effects of inhibition of endogenous prostaglandin synthesis on hepatocyte metabolism (7). The mechanism by which these prostaglandins inhibit VLDL secretion is not clear, but comparison with the effects of agents that interfere with cellular $Ca^{2+}$ metabolism, particularly verapamil, suggest that calcium is involved.

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