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EFFECTS OF 8-*N,N*-DIETHYLAMINO-OCTYL-3,4,5-TRIMETHOXYBENZOATE (TMB-8) HCl AND VERAPAMIL ON THE METABOLISM OF FREE FATTY ACID BY HEPATOCYTES*

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Abstract—The influence of calcium antagonists on hepatic lipid metabolism was investigated in freshly dispersed rat hepatocytes incubated with [1-^{14}C]oleate and verapamil or 8-*N,N*-diethylamino-octyl-3,4,5-trimethoxybenzoate (TMB-8). Synthesis of triglyceride was calculated from the specific radioactivity of [1-^{14}C]oleate in extracted total lipid, after separation of each lipid class by thin-layer chromatography. Ketogenesis was measured enzymatically or as the amount of radioactivity incorporated into neutralized acid-soluble extracts. Neither verapamil nor TMB-8 affected triglyceride synthesis. In contrast, TMB-8 and verapamil exerted a concentration-dependent inhibition of ketogenesis, with TMB-8 being more potent than verapamil (inhibition by 50 μM TMB-8 was 73 ± 9% versus 38 ± 2% inhibition by 50 μM verapamil). Increasing the concentrations of calcium (0 to 4.2 mM) or oleate (0 to 2.0 mM) increased the rate of ketogenesis but did not alter the antiketogenic potency of TMB-8 or verapamil, indicating that inhibition of ketogenesis by these drugs was not calcium dependent. Since the calcium antagonists did not affect ketogenesis from octanoic acid, and since carnitine stimulated ketogenesis from [1-^{14}C]oleate by 25% and reversed the antiketogenic effects of TMB-8 and verapamil, it appeared that the two calcium antagonists inhibited ketogenesis by interfering with the activity of the outer mitochondrial carnitine palmitoyltransferase. In assays of the outer carnitine palmitoyltransferase in isolated mitochondria, both TMB-8 and verapamil were inhibitory. TMB-8 was the more potent inhibitor of this enzyme, and carnitine was able to overcome inhibition by each of the inhibitors. These results suggest that verapamil and TMB-8 may inhibit ketogenesis by mechanisms independent of their well known effects on cellular calcium concentrations, and that inhibition may be competitive with respect to carnitine concentration. However, direct inhibition of carnitine palmitoyltransferase may not explain completely the inhibition of ketogenesis by these drugs, since concentrations required for enzyme inhibition were greater than those required for inhibition of ketogenesis in isolated hepatocytes.

It is well established that calcium (Ca^{2+}) is essential for uptake, esterification and oxidation of free fatty acid (FFA‖) by isolated hepatocytes [1–4] and subcellular preparations [4–8]. Soler-Argilaga *et al.* [1, 8, 9] and Ontko and Otto [4] reported that glucagon and cyclic AMP fail to stimulate ketogenesis by liver in a Ca^{2+}-depleted medium. Esterification of FFA by microsomal preparations is also reduced in the absence of Ca^{2+} [8]. Ontko and coworkers [2–4] demonstrated that Ca^{2+} elevates the mitochondrial NADH/NAD^+ ratio concomitantly with increased ketogenesis in intact hepatocytes. Earlier studies by Mellanby and Williamson [10] showed that Ca^{2+} increases endogenous ketogenesis by liver slices from fasted rats but not by liver slices from fed rats.

The advent of Ca^{2+} channel blockers and their increasing prominence in the management of angina pectoris, hypertension and arrhythmias [11, 12] signal a need for further examination of the role of Ca^{2+} in hepatic lipid metabolism. Deposition of Ca^{2+} in vascular smooth muscle cells is a risk factor in development of coronary artery disease and atherosclerosis [13–16], and agents such as EGTA and diphosphonates which chelate Ca^{2+} have been reported to suppress Ca^{2+}-induced atherogenesis [14–16]. Recently, two Ca^{2+} channel-blockers, nifedipine [17] and nicardipine [18], were observed to retard development of atherosclerosis in cholesterol-fed rabbits and rats respectively. In the rabbit, nifedipine does not alter total serum cholesterol, but decreases atherosclerotic aortic lesions and the cholesterol content of aortic plaques [17]. In the rat, nicardipine decreases total serum cholesterol and LDL cholesterol while increasing HDL cholesterol [18]. These effects of the Ca^{2+} channel blockers are sufficiently significant to warrant further investigation of the mechanisms by which Ca^{2+} may regu-

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‖ Abbreviations: FFA, free fatty acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N*-tetraacetic acid; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BSA, bovine serum albumin; VPML, verapamil; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

late or modulate lipid metabolism. We selected the liver as a possible site of drug action because the liver is the primary organ for plasma VLDL production, and also because VLDL is a metabolic precursor of the atherogenic LDL.

In the present study we incubated freshly dispersed rat hepatocytes with [1-^{14}C]oleate and varied concentrations of verapamil or TMB-8. We observed profound inhibition of ketogenesis by the drugs, whereas they exerted no significant effects on triglyceride synthesis.

METHODS

Animals

Male Sprague–Dawley rats weighing 285–330 g (Harlan Industries, Indianapolis, IN) were housed under 12 hr–12 hr light–dark cycle. The rats were allowed to acclimate for 7 days to the animal facility environment before being killed. Except when indicated otherwise, the rats were fed *ad lib.*

Preparation of hepatocytes

(A) *Cell-dispersion medium.* Ca^{2+}-free Krebs–Henseleit bicarbonate buffer (60 ml) containing 300 mg glucose/dl, and 0.5 mg collagenase (Type IV)/ml, at 37° maintained at pH 7.4 by oxygenation with 95% O_2–5% CO_2 in a recirculating liver perfusion chamber.

(B) *Cell-suspension medium.* Ca^{2+}-free Krebs–Henseleit bicarbonate buffer containing 300 mg glucose/dl (for initial three washes) and 100 mg glucose/dl (for final suspension of washed hepatocytes).

Hepatocytes were prepared by a modification of the method of Berry and Friend [19] using the above cell-dispersion and cell-suspension buffers. Rats were anesthetized with pentobarbital (50 mg/kg). Livers were removed surgically [20] and were perfused in a recycling system [20, 21] with cell dispersion medium A for 20–30 min.

Incubation of hepatocytes

Incubation media. (A) Krebs–Henseleit bicarbonate buffer adjusted to give a final concentration of 2.1 mM Ca^{2+}, 50 mg glucose/dl, 1.0 mM [1-^{14}C]oleic acid plus various amounts (0, 5, 10, 50, 100, or 250 μM) of either verapamil or TMB-8. (B) Ca^{2+}-free Krebs–Henseleit bicarbonate buffer adjusted to give a final concentration of 50 mg glucose/dl, 1.0 mM [1-^{14}C]oleic acid plus various concentrations of Ca^{2+} (0, 0.2, 1.0, 2.1 or 4.2 mM). In some cases the Ca^{2+}-free medium contained 2.0 mM EGTA. A complex of oleate and bovine serum albumin was prepared as described by Van Harken *et al.* [21]. The albumin concentration was 3.0% in all incubation flasks.

A 2-ml sample of freshly dispersed hepatocytes was incubated with 2.0 ml of the different incubation media outlined above. Following incubation for 40 min, the experiment was terminated by transferring 2.0 ml of cell suspension (cells plus incubation medium) into 13.0 ml of methanol for lipid extraction [22], and the remaining cell suspension was deproteinized with 0.5 ml of 15% $HClO_4$. The extracted lipid was separated by thin-layer chromatography [23], and the bands corresponding to free fatty acid and triglyceride were scraped into scintillation vials. Scintillation fluor (10.0 ml of Biocount; Research Products International Corp., Mount Prospect, IL) was added to each sample, and radioactivity was counted in a liquid scintillation spectrometer with a data reduction package (Beckman LD 7500). The $HClO_4$-deproteinized aliquots were neutralized to a methyl orange end-point with 10% KOH. After centrifugation, an aliquot (0.1 ml) of the supernatant fraction was treated with Biocount, and radioactivity was measured by scintillation spectrometry. The radioactivity in the neutralized acid-soluble extracts represents primarily the rate of ketogenesis by the hepatocytes as reported by Stakkestad and Bremer [24]. The incorporation of [1-^{14}C]oleate into triglyceride and the acid-soluble extract was expressed per microgram DNA. The DNA was measured by the spectrofluorometric method described by Fiszer-Szafarz *et al.* [25].

Assay of carnitine palmitoyltransferase

Carnitine palmitoyltransferase was assayed in intact isolated mitochondria as described previously [26]. Mitochondria were isolated by the method of Johnson and Lardy [27] from livers of rats fed *ad lib.* The assay mixture contained, in a total volume of 1.0 ml, 82 mM sucrose, 72 mM HCl, 25 mM HEPES, 35 mM imidazole, 5 mM reduced glutathione, 2 mg bovine serum albumin, 0.5 or 0.1 mM L-carnitine (0.4 μCi of L-[*methyl*-^{3}H]carnitine), 1 μg antimycin A, 2 mM ATP, 2 mM $MgCl_2$, 100 μM palmitoyl-CoA and 0.2 mg of mitochondrial protein. Reactions were carried out at pH 7 and 30° for 5 min following a 5-min preincubation period in the presence of all components except carnitine, which was added to initiate the reaction.

Statistical analysis

Statistical analysis of differences between groups was computed as necessary using Student's *t*-test.

RESULTS

Effects of concentration of Ca^{2+} *on the modulation by TMB-8 and verapamil of the incorporation of* [1-^{14}C]*oleate into triglyceride and ketone bodies*

In agreement with previous data from this laboratory [1], the incorporation by isolated hepatocytes of [1-^{14}C]oleate into triglyceride and ketone bodies was depressed by the omission of Ca^{2+} from the incubation medium. Optimal incorporation of [1-^{14}C]oleate into triglyceride and ketone bodies, determined in preliminary experiments, was observed at an extracellular Ca^{2+} concentration of 2.1 mM (Tables 1 and 2). Omission of Ca^{2+} from the incubation medium, as well as addition of 2.0 mM EGTA to the Ca^{2+}-free incubation medium, reduced ketogenesis and triglyceride synthesis to an equal extent, to about half of that in the complete medium. When the Ca^{2+} concentration was increased to 4.2 mM, the basal rates of ketogenesis and triglyceride synthesis were not changed. Verapamil (50 μM) did not alter significantly the incorporation of [1-^{14}C]oleate into triglyceride at 2.1 and 4.2 mM Ca^{2+}, but resulted in

Table 1. Effects of Ca^{2+} on the modulation by TMB-8 and verapamil of incorporation of [1-^{14}C]oleate into triglyceride

$[Ca^{2+}]$ (mM)	Triglyceride synthesis (nmol [1-^{14}C]oleate/μg DNA/40 min)		
		Experimental	
	Control	50 μM TMB-8	50 μM VPML
1. 0	1.47 ± 0.16 (100)	1.73 ± 0.21 (117 ± 5)	1.04 ± 0.11 (71 ± 5)
2. 0 mM Ca^{2+} +2.0 mM EGTA	1.25 ± 0.38 (100)	1.49 ± 0.47 (119 ± 3)	0.98 ± 0.32 (78 ± 4)
3. 2.1	2.37 ± 0.10 (100)	3.22 ± 0.54* (136 ± 8)	2.14 ± 0.29 (91 ±12)
4. 4.2	2.23 ± 0.26 (100)	2.40 ± 0.45 (108 ± 10)	1.96 ± 0.31 (88 ± 5)

Freshly dispersed hepatocytes were incubated with BSA–[1-^{14}C]oleate complex in Krebs–Henseleit bicarbonate buffer containing 0 to 4.2 mM final concentrations of Ca^{2+} and 1.0 mM oleate. At each Ca^{2+} concentration, and in the presence of 2.0 mM EGTA, the vessels contained no drug, 50 μM TMB-8 or 50 μM verapamil. TG synthesis was measured as described in the text. Each value represents the mean ± SE for four or more separate hepatocyte preparations. Values in parentheses are percent of control.
* Statistical significance of difference due to drug effect: $P < 0.05$.

a decrease (about 30% reduction) in the Ca^{2+}-free medium. TMB-8, on the other hand, stimulated triglyceride synthesis at 2.1 mM Ca^{2+} (50% increase), but it had no effect at other concentrations of Ca^{2+} examined. As shown in Table 2, verapamil and TMB-8 exerted relatively constant inhibitory effects on the incorporation of [1-^{14}C]oleate into acid-soluble radioactivity in the presence of various concentrations of Ca^{2+}. Thus, in the incubation media containing 0, 2.1 and 4.2 mM Ca^{2+}, TMB-8 depressed ketogenesis to about 25% of control, whereas verapamil depressed it to approximately 60%.

Effects of concentration of verapamil and TMB-8 on metabolism of [1-^{14}C]Oleate

The incorporation of [1-^{14}C]oleate into triglyceride was not altered by 10–250 μM verapamil (Fig. 1). However, at a concentration of 500 μM, verapamil depressed triglyceride synthesis to 47.1 ± 5.1% of control (data not shown). In contrast, 10–100 μm TMB-8 tended to increase triglyceride synthesis at low concentrations, albeit not statistically significant. At 250 μM, the synthetic rate returned to basal levels, while at 500 μM TMB-8 there was a depression (58.8 ± 10.6% control) of incorporation of [1-^{14}C]oleate into triglyceride (data for 500 μM not shown in figure). Despite the lack of stimulation by verapamil and TMB-8 of triglyceride synthesis, both drugs depressed ketogenesis. TMB-8 was a more potent antiketogenic agent than verapamil. In one group of hepatocyte preparations, 50 μM TMB-8 depressed ketogenesis to 25.9 ± 2.7% of control,

Table 2. Effects of Ca^{2+} on the modulation by TMB-8 and verapamil of incorporation of [1-^{14}C]oleate into ketone bodies

$[Ca^{2+}]$ (mM)	Ketone body production (nmol [1-^{14}C]oleate/μg DNA/40 min)		
		Experimental	
	Control	50 μM TMB-8	50 μM VPML
1. 0	0.85 ± 0.08 (100)	0.21 ± 0.02 (25 ± 2)	0.58 ± 0.05 (68 ± 1)
2. 0 mM Ca^{2+} +2.0 mM EGTA	0.67 ± 0.10 (100)	0.18 ± 0.03 (27 ± 3)	0.40 ± 0.06 (60 ± 2)
3. 2.1	1.20 ± 0.09 (100)	0.25 ± 0.03 (21 ± 2)	0.65 ± 0.05 (54 ± 3)
4. 4.2	1.03 ± 0.10 (100)	0.23 ± 0.04 (22 ± 2)	0.67 ± 0.08 (65 ± 3)

Experimental conditions were the same as described in Table 1. After removal of the aliquot for lipid extraction, the remaining cell suspension was deproteinized by 15% $HClO_4$, and the neutralized acid-soluble extract was used to compute the rate of ketogenesis. Values are means ± SE for four or more separate hepatocyte preparations. At each concentration of Ca^{2+}, the effects of TMB-8 and verapamil differed significantly from control at $P < 0.005$. Values in parentheses are percent of control.

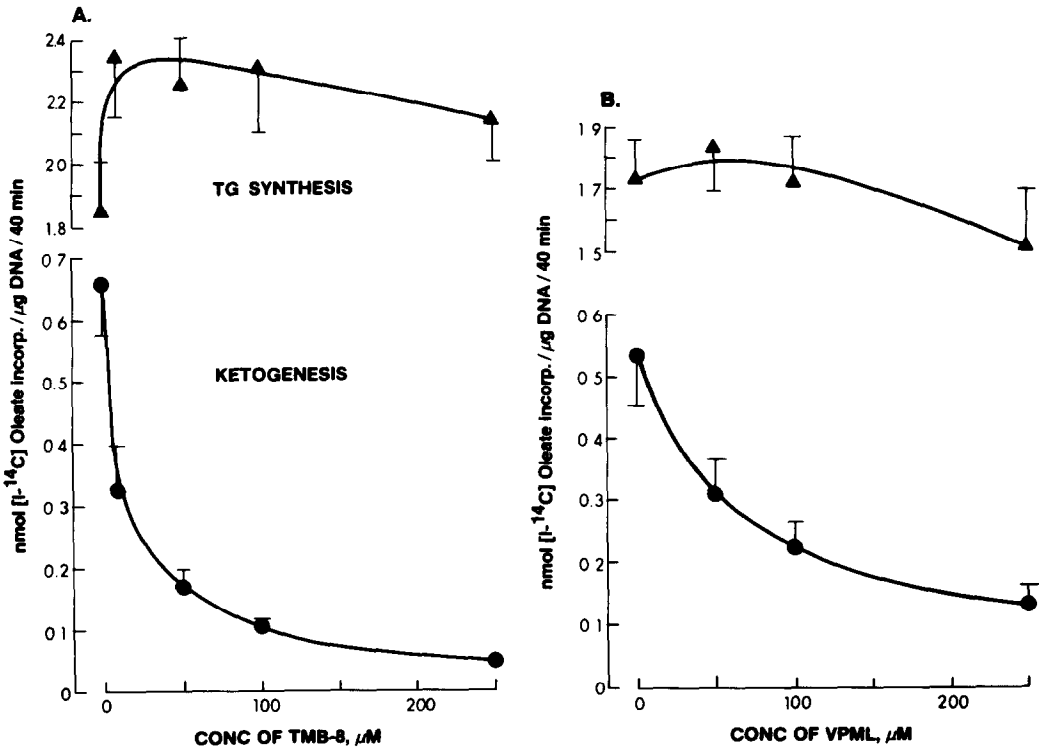

Fig. 1. Effects of TMB-8 and verapamil on metabolism of [1-^{14}C]oleic acid by isolated hepatocytes. Hepatocytes were incubated with 1.0 mM [1-^{14}C]oleate in Krebs–Henseleit bicarbonate buffer containing 2.1 mM Ca^{2+} and various concentrations (0–250 μM) of TMB-8 or verapamil. Triglyceride synthesis and ketogenesis were estimated as described in the text. Panel A shows effects of TMB-8; panel B shows effects of verapamil. Each point represents the mean ± SE for six or more separate hepatocyte preparations. Upper panel: TG synthesis. Lower panel: ketogenesis.

whereas 50 μM verapamil depressed it to 58.0 ± 2.4%.

Effects of concentration of [1-^{14}C]oleate on the modulation by verapamil and TMB-8 of incorporation into triglyceride and ketone bodies

As expected, increasing concentration of oleic acid stimulated incorporation of [1-^{14}C]oleate into both triglyceride and neutralized acid-soluble extracts. As outlined in Table 3, verapamil and TMB-8 (50 μM) had no effects on the incorporation into triglyceride at any concentration of oleate. The depression of ketogenesis by both TMB-8 and verapamil was not altered by increasing concentrations of [1–^{14}C]oleate. Thus, at all oleate concentrations, 50 μM TMB-8 reduced ketogenesis by about 75% while 50 μM verapamil inhibited by about 25% (Table 4).

Effects of TMB-8 and verapamil on ketone body production from oleic acid

Enzymatically measured, the rate of basal production of acetoacetate and β-hydroxybutyrate at a concentration of 1.0 mM oleate were, respectively, 4.0 ± 0.1 and 5.2 ± 0.5 nmol/μg DNA/40 min, and the ratio β-hydroxybutyrate/acetoacetate was 1.3 ± 0.1 (Table 5). In the presence of 50 μM TMB-8, these values were reduced significantly; 50 μM verapamil was somewhat less potent, decreasing the production of each of the ketone bodies by 35% to 2.6 ± 0.2 and 3.3 ± 0.4 nmol/μg DNA/40 min, respectively, for acetoacetate and β-hydroxybutyrate, and having no effect on the ratio. The inhibition of ketone body production by the two drugs measured by radioactive incorporation of [1-^{14}C]oleate was in close agreement with that measured enzymatically.

A submaximal concentration of TMB-8 (20 μM) produced about 47% reduction in the level of both acetoacetate and β-hydroxybutyrate. However, when 20 μM TMB-8 was combined with 50 μM verapamil, acetoacetate production was reduced to 1.8 ± 0.1 nmol/μg DNA/40 min, a 55% reduction, whereas β-hydroxybutyrate production dropped by 83% to 0.9 ± 0.2 nmol/μg DNA/40 min. Thus, an additive effect of the two drugs was shown on β-hydroxybutyrate production, but their effect on acetoacetate was not additive. Neither TMB-8 (20 μM) nor verapamil (50 μM) exerted any alteration on the ratio β-hydroxybutyrate/acetoacetate, but the combination of the drugs reduced the ratio markedly. The effects of the combination of the drugs on incorporation of [1-^{14}C]oleate into neutralized acid-soluble extracts were similar to their additive effects on the β-hydroxybutyrate production (Table 6).

Table 3. Influence of oleate concentration on the effects of TMB-8 and verapamil on incorporation of [1-^{14}C]oleate into triglyceride by rat hepatocytes

[Oleate] (mM)	Triglyceride synthesis (nmol [1-^{14}C]oleate/μg DNA/40 min)		
	Control	Experimental	
		50 μM TMB-8	50 μM VPML
1. 0.2	0.55 ± 0.07 (100)	0.62 ± 0.06 (113 ± 8)	0.63 ± 0.09 (115 ± 14)
2. 0.5	1.26 ± 0.12 (100)	1.52 ± 0.08 (121 ± 15)	1.34 ± 0.12 (106 ± 3)
3. 1.0	2.11 ± 0.18 (100)	2.54 ± 0.22 (120 ± 11)	2.34 ± 0.17 (111 ± 6)
4. 2.0	3.42 ± 0.33 (100)	3.87 ± 0.40 (113 ± 4)	3.71 ± 0.29 (108 ± 3)

Hepatocytes were incubated with BSA-[1-^{14}C]oleate complex at final concentrations of 0.2, 0.5, 1.0 and 2.0 mM in Krebs–Henseleit bicarbonate buffer. Data are means ± SE for five separate hepatocyte preparations. At each oleate concentration, incubation vessels contained no drug, 50 μM TMB-8 or 50 μM verapamil. Triglyceride synthesis was estimated as described in the text. Values in parentheses are percent of control.

Effects of TMB-8 and verapamil on ketone body production from octanoate

The basal rates of production of acetoacetate and β-hydroxybutyrate from octanoate (1.0 mM) were, respectively, 3.7 ± 0.3 and 7.6 ± 1.4 nmol/μg DNA/40 min (Table 7). In contrast to effects on oxidation of oleate, TMB-8 at a concentration of 50 μM did not alter production of acetoacetate or β-hydroxybutyrate from octanoate; 50 μM verapamil produced a slight, but statistically insignificant, reduction of the rates of ketogenesis.

Reversal of antiketogenic effects of TMB-8 and verapamil by carnitine

Addition of L-carnitine (0.5 mM) to the incubation medium induced a 25% increase in rates of ketogenesis from [1-^{14}C]oleate by freshly dispersed hepatocytes (1.41 ± 0.14 vs 1.15 ± 0.13 nmol oleate incorporated/μg DNA/40 min, Fig. 2). The ketogenic rate was depressed by TMB-8 (50 μM) to 25.8 ± 1.4% of control, whereas 50 μM verapamil depressed it to 57.4 ± 5.6% of control. In the presence of L-carnitine, TMB-8 (50 μM) and verapamil (50 μM) were less antiketogenic, as they reduced ketogenesis to only 67.1 ± 4.8 and 84.9 ± 3.7% of control respectively.

Effects of TMB-8, verapamil and Ca^{2+} on relative utilization of [1-^{14}C]oleate for ketogenesis and triglyceride synthesis

The ratio of [1-^{14}C]oleate incorporated into TG/[1-^{14}C]oleate incorporated into ketone bodies is

Table 4. Influence of oleate concentration on the effects of TMB-8 and verapamil on incorporation of [1-^{14}C]oleate into ketone bodies by rat hepatocytes

[Oleate] (mM)	Ketone body production (nmol [1-^{14}C]oleate/μg DNA/40 min)		
	Control	Experimental	
		50 μM TMB-8	50 μM VPML
1. 0.2	0.25 ± 0.02 (100)	0.09 ± 0.02 (36 ± 6)	0.16 ± 0.02 (64 ± 6)
2. 0.5	0.74 ± 0.14 (100)	0.22 ± 0.04 (30 ± 5)	0.46 ± 0.10 (62 ± 4)
3. 1.0	1.10 ± 0.19 (100)	0.28 ± 0.05 (26 ± 3)	0.65 ± 0.13 (59 ± 2)
4. 2.0	1.48 ± 0.32 (100)	0.40 ± 0.07 (27 ± 2)	0.91 ± 0.19 (61 ± 3)

Experimental conditions were the same as in Table 3. Ketogenesis was measured as outlined in the text. Values are means ± SE for five separate hepatocyte preparations at each concentration of oleate. The effects of TMB-8 differed significantly from controls at $P < 0.005$, whereas those of verapamil were significant at $P < 0.05$. Values in parentheses are percent of control.

Table 5. Effects of TMB-8 and verapamil on synthesis of acetoacetate and β-hydroxybutyrate by hepatocytes

Additions	Ketone body production (nmol/μg DNA/40 min) (a) Acetoacetate	(b) β-Hydroxybutyrate	$\frac{b}{a}$
1. 1.0 mM Oleate	4.03 ± 0.09 (100)	5.21 ± 0.53 (100)	1.30 ± 0.13
2. 1.0 mM Oleate +20 μM TMB-8	2.17 ± 0.04 (54 ± 2)	2.76 ± 0.04 (53 ± 3)	1.27 ± 0.12
3. 1.0 mM Oleate +50 μM TMB-8	1.76 ± 0.07 (44 ± 3)	1.68 ± 0.19 (32 ± 1)	0.95 ± 0.12
4. 1.0 μM Oleate +50 μM VPML	2.63 ± 0.22 (65 ± 6)	3.34 ± 0.43 (64 ± 4)	1.27 ± 0.22
5. 1.0 mM Oleate +20 μM TMB-8 +50 μM VPML	1.81 ± 0.12 (45 ± 3)	0.89 ± 0.22 (17 ± 4)	0.49 ± 0.16

Hepatocytes were incubated with a BSA–1.0 mM [1-^{14}C]oleate complex in Krebs–Henseleit bicarbonate buffer containing (1) no drug, (2) 20 μM TMB-8, (3) 50 μM TMB-8, (4) 50 μM verapamil or (5) a combination of 20 μM TMB-8 with 50 μM verapamil. Ketone bodies formed were measured enzymatically as described in the text. Values shown are means ± SE for five separate hepatocyte preparations. Inhibition of ketogenesis by verapamil, TMB-8 or drug combination was statistically significant at $P < 0.005$. Numbers in parentheses are percent of control.

Table 6. Effect of combination of TMB-8 with verapamil on incorporation of [1-^{14}C]oleate into neutralized acid-soluble extracts

Additions	Ketogenesis (nmol [1-^{14}C]oleate/μg DNA/40 min)
1. 1.0 mM Oleate	1.10 ± 0.19 (100)
2. 1.0 mM Oleate +20 μM TMB-8	0.44 ± 0.15 (40 ± 6)
3. 1.0 mM Oleate 50 μM VPML	0.65 ± 0.13 (59 ± 2)
4. 1.0 mM Oleate +20 μM TMB-8 +50 μM VPML	0.25 ± 0.06 (23 ± 2)

Experimental conditions were the same as in Table 5. The rate of ketogenesis was computed from oleate radioactivity incorporated into the neutralized acid-soluble extract. Values in parentheses are percent of control.

Table 7. Effects of TMB-8 and verapamil on the oxidation of octanoic acid to ketone bodies by hepatocytes

Additions	Ketone bodies produced (nmol/μg DNA/40 min) (a) Acetoacetate	(b) β-Hydroxybutyrate	$\frac{b}{a}$
1. No drug	3.72 ± 0.34 (100)	7.58 ± 1.39 (100)	2.03 ± 0.20
2. 50 μm TMB-8	3.60 ± 0.38 (97 ± 1)	6.85 ± 1.29 (90 ± 3)	1.90 ± 0.20
3. 50 μM VPML	3.28 ± 0.35 (88 ± 3)	5.41 ± 1.27 (71 ± 4)	1.65 ± 0.20

Incubation conditions and assay procedures were similar to those described in the legend to Table 5, except that 1.0 mM octanoic acid was the substrate for ketogenesis, instead of oleic acid. Data are means ± SE for five separate hepatocyte preparations. Values in parentheses are percent of control.

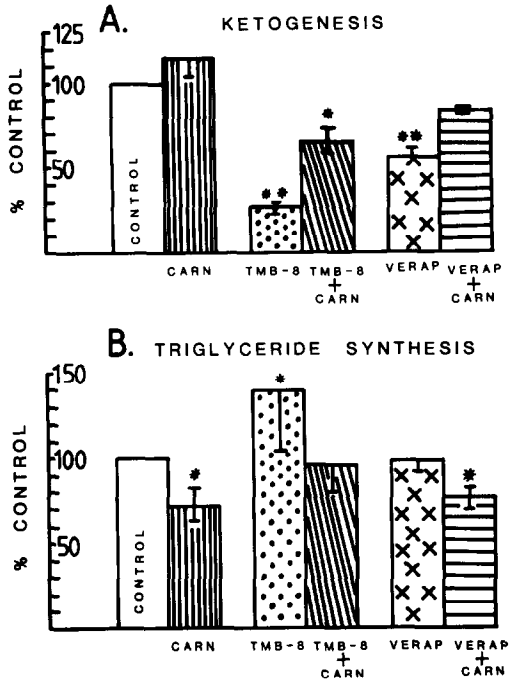

Fig. 2. Effects of TMB-8 and verapamil on metabolism of [1-^{14}C]oleate by isolated hepatocytes in the absence and presence of L(−)carnitine. Hepatocytes were incubated with 1.0 mM oleate with or without 0.5 mM (−)carnitine. Some of the cells to which carnitine was added or omitted were also treated with 50 μM TMB-8 or 50 μM verapamil. TG synthesis and ketogenesis were estimated as described in the text. Each bar represents percent of control mean ± SE for four separate hepatocyte preparations. Control values for ketogenesis: 1.15 ± 0.13 nmol [1-^{14}C]oleate incorporated/μg DNA/40 min; control values for TG synthesis: 2.3 ± 0.1 nmol [1-^{14}C]oleate incorporated/μg DNA/40 min. Statistical significance of drug effects: (*P) < 0.05; and (**) P < 0.005.

shown in Fig. 3. This ratio measures the relative magnitude of activities along the synthetic pathways for triglyceride and ketone bodies in the presence of various concentrations of TMB-8, verapamil and Ca^{2+}. The ratio for Ca^{2+} was flat, indicating that, at all Ca^{2+} concentrations examined, Ca^{2+} exerted identical effects on oleate incorporation into TG and ketone bodies. Increasing the concentration of TMB-8 or verapamil increased the incorporation ratio, reflecting the inhibition by these drugs of ketogenesis.

Inhibition of carnitine palmitoyltransferase by TMB-8 and verapamil

The outer carnitine palmitoyltransferase, assayed in intact, isolated mitochondria was inhibited by both TMB-8 and verapamil (Fig. 4). TMB-8 was a more potent inhibitor of this enzyme than verapamil, but higher concentrations of each of the drugs were required for enzyme inhibition in isolated mitochondria than for a similar percentage inhibition of ketogenesis in isolated hepatocytes. Inhibition of carnitine palmitoyltransferase by both inhibitors was much greater at a lower concentration of carnitine, in agreement with the effects of carnitine in isolated hepatocytes.

DISCUSSION

The data presented constitute potentially important information about metabolic effects of Ca^{2+} channel blockers. The data confirm earlier observations from this laboratory [1, 8, 9] and others [2, 7] that Ca^{2+} is essential for metabolism of FFA by hepatocytes. The Ca^{2+} antagonists used in these studies allows blockade of Ca^{2+} fluxes at two different sites; verapamil presumably acts to block influx of extracellular Ca^{2+} [11, 12], whereas TMB-8 blocks intracellular movement of Ca^{2+} [28]. These differential effects on Ca^{2+} fluxes may explain why TMB-8 consistently exerted a more inhibitory effect than did verapamil on ketogenesis. It may be inferred from this observation that the intracellular fluxes of Ca^{2+}, probably those regulated by the mitochondria, are more important than influx of extracellular Ca^{2+} for the control of ketogenesis by Ca^{2+}.

It is logical to speculate that the antiketogenic effects of TMB-8 and verapamil are mediated through blockade of Ca^{2+} movement. Hence, it might have been expected that the antiketogenic effects would be enhanced by low Ca^{2+} concentrations, whereas higher concentrations of Ca^{2+} should reduce the effects. Our data did not bear out this expectation. In fact, the inhibitory effects of TMB-8 and verapamil were about the same in extracellular concentrations of 0, 2.1 and 4.2 mM Ca^{2+}. Furthermore, the curve for the effect of Ca^{2+} on the ratio of [1-^{14}C]oleate esterified/oxidized was very different from the curves of the effects of TMB-8 and verpamil. Thus, it is possible that the effects of TMB-8 and verapamil may be mediated, in part, through direct actions and not entirely through modification of Ca^{2+} fluxes.

To elucidate the site along the ketogenic pathway at which TMB-8 and verapamil might exert their antiketogenic effects, we examined the interaction of TMB-8 and verapamil with increasing oleate concentration, carnitine, and octanoate. Since ketogenesis increases as oleate concentration increases [22, 29–33], the possibility existed that the inhibitory effects of verapamil and TMB-8 would be reversed by increasing concentrations of oleate. Such was not the case in this study. Although rates of ketogenesis increased with oleate concentration, the percentage inhibition of ketogenesis by 50 μM TMB-8 (26%) and that by 50 μM verapamil (58% of control) was not altered when the drugs were incubated with 0.2, 0.5 or 2.0 mM oleate. These observations suggested that the inhibitory effects of TMB-8 and verapamil on ketogenesis were not mediated through blockade of free fatty acid uptake. Furthermore, the lack of effect of these two drugs on ketogenesis when octanoate was the substrate strongly suggested that enzymes of β-oxidation were not affected. The observation that L(−)carnitine partially reversed the inhibitory effect of TMB-8 and almost completely reversed that of verapamil is of particular interest. These observations suggested that TMB-8 and verapamil might compete with carnitine at the level of the transport of long chain acyl-CoA across the

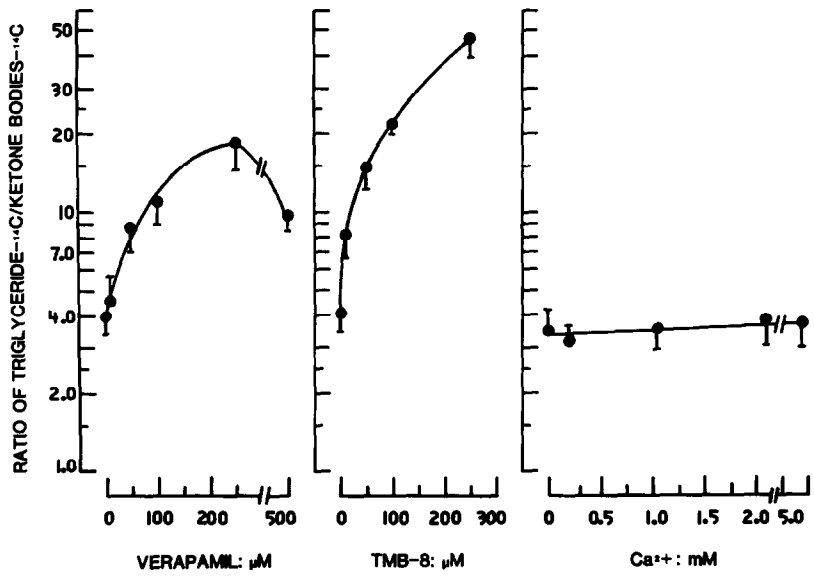

Fig. 3. Effects of TMB-8, verapamil and Ca^{2+} on relative utilization of [1-^{14}C]oleate for ketogenesis and synthesis of triglyceride. Hepatocytes were incubated with 1.0 mM [1-^{14}C]oleate in Krebs–Henseleit bicarbonate buffer containing 2.1 mM Ca^{2+} and various concentrations of TMB-8 or verapamil. Other hepatocytes were incubated with various concentrations of Ca^{2+}, in the absence of drug, but with 1.0 mM oleate. The ratio of esterified/oxidized oleate was computed from:

$$\frac{\text{nmol [1-}^{14}\text{C]oleate incorporated into TG/}\mu\text{g DNA/40 min}}{\text{nmol[1-}^{14}\text{C]oleate incorporated into ketones/}\mu\text{g DNA/40 min}}$$

Each point represents the mean ± SE for six or more separate hepatocyte preparations.

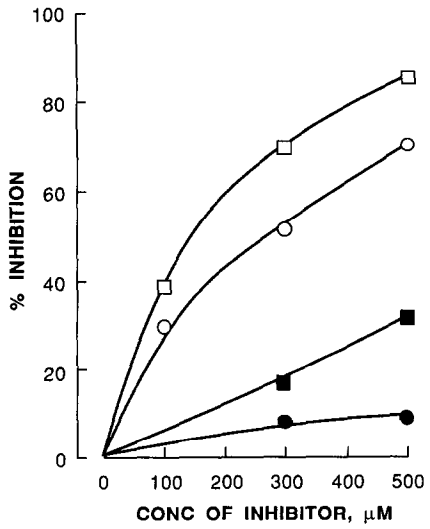

Fig. 4. Inhibition of carnitine palmitoyltransferase by TMB-8 and verapamil. The outer carnitine palmitoyltransferase was assayed in intact, isolated mitochondria at either 0.1 mM carnitine (open symbols) or at 0.5 mM carnitine (closed symbols) in the absence or presence of various concentrations of TMB-8 (□, ■) or verapamil (○, ●). Each point represents the average of two separate experiments using mitochondria isolated from different animals. The specific activity of the outer carnitine palmitoyltransferase measured at optimal concentrations of 0.5 mM carnitine and 0.1 mM palmitoyl-CoA was 8.8 ± 0.5 nmol/min/mg protein (mean ± SE, N = 4).

mitochondrial inner membrane [33–38]. This possibility was further supported by the fact that these two drugs had negligible effects on ketogenesis by hepatocytes when octanoate was the substrate, since octanoate does not require the action of carnitine palmitoyltransferase to traverse the mitochondrial inner membrane. Studies of the inhibition of carnitine palmitoyltransferase by TMB-8 and verapamil confirmed that both drugs were direct inhibitors of this enzyme. Since TMB-8 was a more potent inhibitor than verapamil and since both inhibitors were less effective at higher concentrations of carnitine, direct inhibition of carnitine palmitoyltransferase by these drugs may explain much, if not all, of their effects on fatty acid metabolism. The fact that more of each drug was required to inhibit carnitine palmitoyltransferase than was required for similar levels of inhibition of ketogenesis in isolated hepatocytes may suggest that additional effects of these drugs exist or that the conditions in the enzyme assay were not exactly the same as those in isolated hepatocytes. Whether TMB-8 and verapamil might act through an additional mechanism involving control of intracellular calcium levels cannot be assessed accurately at this time, but the fact that both drugs had effects in the absence of extracellular calcium suggests that calcium is not involved as a major mechanism of drug action. The antagonism by carnitine of the actions of these drugs suggests that they act by a mechanism similar to *p*-hydroxyphenylglyoxylate, the active metabolite of oxfenicine, which is competitive with carnitine [39]. Malonyl-CoA, the

physiological inhibitor of carnitine palmitoyltransferase, is a competitive inhibitor with respect to the acyl-CoA substrate and noncompetitive with respect to carnitine [40].

The site of action of both TMB-8 and verapamil was examined further by combining the two drugs. The sum of individual effects of 20 μM TMB-8 and 50 μM verapamil was equal to the effect produced by the simultaneous presence of the two drugs in the incubation medium. This additive effect was observed for incorporation of [1-^{14}C]oleate into acid-soluble extracts and production of β-hydroxybutyrate, but not for acetoacetate production. The significance of this observation was not apparent from our study. Nevertheless, it may indicate that although TMB-8 acts intracellularly on Ca^{2+} movements, while verapamil acts on slow Ca^{2+} channels on the plasma membrane, both drugs blocked ketogenesis at the same site, presumably carnitine palmitoyltransferase. This suggests that, even though fatty acid oxidation may be stimulated by Ca^{2+}, TMB-8 and verapamil may inhibit fatty acid oxidation by a mechanism not involving Ca^{2+}.

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