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REDUCTION OF RAT LIVER ENDOPLASMIC RETICULUM Ca^{2+}-ATPase ACTIVITY AND MOBILIZATION OF HEPATIC INTRACELLULAR CALCIUM BY CIPROFIBRATE, A PEROXISOME PROLIFERATOR

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Abstract—Ciprofibrate, a peroxisome proliferating agent, induces cell proliferation in rodent liver during the early periods of exposure. Since Ca^{2+} plays an important role in mitogenesis, we have investigated the effects of ciprofibrate on hepatic endoplasmic reticulum (ER) Ca^{2+}-ATPase, which in part regulates Ca^{2+} homeostasis. A single oral dose of 200 mg/kg ciprofibrate to male F344 rats produced a transient decrease in liver microsomal Ca^{2+}-ATPase activity to 48% of control levels at 24 hr post-exposure. Activity had returned to control levels by 48 and 72 hr after exposure. The decrease in Ca^{2+}-ATPase activity was not a function of non-specific enzymatic inhibition, since activity of another microsomal enzyme, glucose-6-phosphatase, was not altered in ciprofibrate-exposed rats. Using an ATP-driven $^{45}Ca^{2+}$ accumulation assay, rats exposed to 25, 100 and 200 mg/kg ciprofibrate exhibited a dose-dependent inhibition of liver microsomal Ca^{2+} accumulation at 24 hr post-exposure. Analysis of Western immunoblots using a polyclonal antibody to the liver ER Ca^{2+}-ATPase revealed a marginal increase in Ca^{2+}-ATPase protein content in microsomes prepared from ciprofibrate-exposed rats compared to controls 24 hr post-exposure. These data indicate that the reduction of Ca^{2+}-ATPase activity is not attributable to diminished Ca^{2+}-ATPase protein content *in vivo* and, therefore, is due to a functional inhibition of the enzyme. Ciprofibrate also produced a concentration-dependent inhibition of rat liver ER Ca^{2+}-ATPase activity *in vitro* ($IC_{50} \approx 170\ \mu M$). In freshly isolated rat hepatocytes, ciprofibrate elevated the free intracellular calcium concentration ($[Ca^{2+}]_i$) in the presence and absence of extracellular calcium. Collectively, these results suggest that ciprofibrate mobilizes hepatic $[Ca^{2+}]_i$ via inhibition of the ER Ca^{2+}-ATPase. These events may lead to an environment of elevated $[Ca^{2+}]_i$ during the early stages of ciprofibrate exposure and may serve to augment Ca^{2+}-dependent processes, thus playing a pivotal role in the acute mitogenic response.

Ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)-phenoxy]-2-methylpropanoic acid) is a non-genotoxic hepatocarcinogen which induces peroxisome proliferation in rodent liver [1–3]. Peroxisome proliferating agents (PPAs†) such as ciprofibrate, clofibrate and nafenopin produce a variety of metabolic alterations in rodent liver, including the induction of peroxisomal β-oxidation enzymes, catalase and carnitine acyltransferase [4, 5], induction of P-450IVA1 [6] and alkaline phosphatase [7], and inhibition of other enzyme systems such as γ-glutamyltranspeptidase [7], glutathione peroxidase and glutathione-*S*-transferase [8]. Another feature of ciprofibrate and several other PPAs is that these agents behave as potent hepatic mitogens. A single exposure to a PPA induces both hypertrophy and hyperplasia in livers of rats and mice [1–3]. This mitogenic stimulus leads to enhanced DNA synthesis occurring both *in vivo* and in cultured hepatocytes within 2–3 days following PPA exposure [9, 10], and subsiding rapidly thereafter.

The mechanism(s) of PPA-induced mitogenicity remains unknown. Elevation of free intracellular calcium concentration ($[Ca^{2+}]_i$) can serve as a mitogenic signal in many cell systems including hepatocytes [11, 12]. To determine if the PPAs could affect hepatic Ca^{2+} homeostasis, we have investigated the effects of ciprofibrate on endoplasmic reticulum (ER) Ca^{2+}-ATPase. This enzyme functions to sequester Ca^{2+} back into the ER [13], thus serving as an important component in attenuating the effects of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) Ca^{2+} mobilization [14]. Although mitochondria have been regarded previously as important in the regulation of $[Ca^{2+}]_i$, they have a low affinity for Ca^{2+} ($K_m \approx 10\ \mu M$) [15] compared to the ER Ca^{2+}-ATPase which is a high affinity ($K_m \approx 0.2\ \mu M$), low capacity (≈ 16 nmol Ca^{2+}/mg protein), Mg^{2+}-ATP dependent Ca^{2+} transporter [13]. Furthermore, studies have demonstrated that mitochondria contain little Ca^{2+} *in situ* [16] and hence the role for mitochondria appears to be inconsistent with a buffering capacity at physiological $[Ca^{2+}]_i$ [15]. It is

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† Abbreviations: PPA, peroxisome proliferating agent; ER, endoplasmic reticulum; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; SR, sarcoplasmic reticulum; tBuHQ, 2,5-di-(*tert*)-butylhydroquinone; TCA, trichloroacetic acid; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; DMSO, dimethyl sulfoxide; and PBS, phosphate-buffered saline.

generally accepted, therefore, that the ER is the major regulator of $[Ca^{2+}]_i$ and, thus, the Ca^{2+} regulating components of this system.

During preparation of this paper Ochsner *et al.* [17] showed that the PPA nafenopin increases hepatocyte $[Ca^{2+}]_i$ independently of Ins(1,4,5)P_3 metabolism. Ciprofibrate and clofibrate were also reported to increase hepatic $[Ca^{2+}]_i$. We now corroborate these findings and demonstrate that ciprofibrate also reduces rat liver ER Ca^{2+}-ATPase activity *in vivo* and *in vitro*. Thus, elevation of hepatic $[Ca^{2+}]_i$ by ciprofibrate may be attributed to the inhibition of Ca^{2+} resequestration, as observed with other ER Ca^{2+}-ATPase inhibitors [14, 18]. These data further suggest that the liver ER Ca^{2+}-ATPase activity could be sufficiently depressed *in vivo* by ciprofibrate to create an environment of elevated $[Ca^{2+}]_i$ during the early periods of exposure which could ultimately contribute to the initiation of an acute mitogenic response.

MATERIALS AND METHODS

Materials and animals. Collagenase (type I) and all other enzyme assay materials were obtained from the Sigma Chemical Co. (St. Louis, MO). Adenosine 5′-trisphosphate (ATP) and [Arg^8] vasopressin were obtained from Boehringer Mannheim (Indianapolis, IN), and 2,5-di-(*tert*)-butylhydroquinone (tBuHQ) was from the Aldrich Chemical Co. (Milwaukee, WI). $^{45}CaCl_2$ was supplied by the Amersham Corp. (Arlington Heights, IL). The cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase C4 polyclonal antibody (Ab C4) [19] was a gift from Dr. David MacLennan and Dr. Jonathan Lytton. Western blot analysis was carried out using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Indo-1-AM was obtained from Molecular Probes Inc. (Eugene, OR). All other chemicals were of the highest purity commercially available.

Fed male F344 rats obtained from Charles River Laboratories (Kingston, NY) weighing 200–225 g were dosed orally with corn oil as control (0.27 mL/100 g body wt) or ciprofibrate (Sterling–Winthrop) in a corn oil vehicle (0.27 mL/100 g body wt) at 25, 100 and 200 mg/kg. Rats were then killed by decapitation at 24, 48 or 72 hr post-treatment. *In vitro* determination of rat liver microsomal Ca^{2+}-ATPase activity and $^{45}Ca^{2+}$ accumulation was carried out by preparing microsomes from livers of fed untreated male F344 rats weighing 200–250 g. For these *in vitro* experiments ciprofibrate was dissolved in a dimethyl sulfoxide (DMSO) vehicle.

Microsomal preparations. Microsomal fractions were prepared essentially as described by Dawson and Fulton [13]. Male F344 rats were killed by decapitation, and livers were removed and immediately placed on ice in a buffer containing 250 mM sucrose, 5 mM HEPES-KOH (pH 7.0), 1 mM dithiothreitol and 0.5 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA). Livers were minced and gently homogenized in a Potter–Elvehjem-type glass/teflon homogenizer (3–4 strokes). Following centrifugation at 1000 *g* for 5 min, the pellet was discarded and the supernatant was centrifuged for a further 10 min at 8000 *g*. The resulting supernatant was removed and centrifuged for 20 min at 35,000 *g*. The pellet was then resuspended in a buffer containing 250 mM sucrose, 5 mM HEPES-KOH (pH 7.0), 100 mM KCl and 1 mM dithiothreitol and recentrifuged at 35,000 *g* for 20 min to yield the heavy microsomal fraction. Protein concentration was determined by a modified Bradford method using the Coomasie Brilliant Blue G-250 solution (Pierce, Rockford, IL) and bovine serum albumin as standard (Pierce, Rockford, IL) [20].

Enzyme assays. All microsomal preparations were analyzed immediately after preparation for either Ca^{2+}-ATPase activity of $^{45}Ca^{2+}$ accumulation. The remaining protein homogenates were frozen at −70° for later measurements of glucose-6-phosphatase and 5′-nucleotidase. Glucose-6-phosphatase was assayed as described by Dawson and Irvine [21]. Briefly, 0.5 mg of microsomal protein was incubated at 37° in a 1.0 mL buffer containing 100 mM KCl, 5 mM HEPES-KOH (pH 7.0) and 5 mM glucose-6-phosphate. After 10 min, the reactions were stopped by the addition of 0.5 mL of 5% trichloroacetic acid (TCA). Released inorganic phosphate (P_i) was measured by the method of Fiske and SubbaRow [22]. 5′-Nucleotidase was measured by a modified method based on Dixon and Purdom [23], which involves the hydrolysis of adenosine 5′-monophosphate to adenosine and P_i. The release of P_i was measured as described by the method of Fiske and SubbaRow [22].

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase activity of rat liver microsomal fractions was determined by the hydrolysis of P_i from ATP in the presence and absence of Ca^{2+} [13]. The incubation medium contained, in a final volume of 1.0 mL; 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES-KOH (pH 7.0), 1 μg oligomycin (mitochondrial Ca^{2+}-ATPase inhibitor) and 1 μg of calcium ionophore A23187 (to prevent rate limitation of Ca^{2+}-ATPase activity). For medium representing Mg^{2+}-ATPase activity, 1 mM EGTA was present, and for the medium representing the Ca^{2+},Mg^{2+}-ATPase activity, 50 μM $CaCl_2$ was present. The medium was allowed to incubate for approximately 5 min at 37° prior to the addition of 1.0 mg of microsomal protein, which was allowed to incubate at 37° for approximately 2 min. The reaction was initiated by the addition of 1 mM ATP, and stopped after 10 min at 37° by the addition of 0.5 mL of 5% TCA. Following centrifugation, 1.0 mL of the supernatant was removed and P_i was determined. The paired difference between P_i hydrolysis with and without Ca^{2+} was representative of Ca^{2+}-ATPase activity.

For *in vitro* experiments, the reaction mixtures were incubated at 37° for 5 min in the presence of various concentrations of ciprofibrate. Microsomal protein (1.0 mg) was added and allowed to incubate at 37° for a further 5 min. Reactions were initiated by the addition of 1 mM ATP, and P_i release was determined after a 10-min incubation. The identity of the ER Ca^{2+}-ATPase was verified by the use of tBuHQ, a specific ER Ca^{2+}-ATPase inhibitor, which does not inhibit either plasma membrane or mitochondrial Ca^{2+} effusion or sequestration [14, 24]. tBuHQ (10 μM) was added to the incubation mixture

containing 1.0 mg protein and incubated at 37° for 5 min prior to the initiation of the reaction with 1 mM ATP.

Microsomal $^{45}Ca^{2+}$ accumulation assay. Accumulation of Ca^{2+} into microsomes was assayed using a filtration method as described by Moore *et al.* [25]. Rat liver microsomes from control and ciprofibrate-exposed rats were prepared as previously described. Microsomes (0.5 mg protein) were incubated for approximately 5 min in an incubation mixture containing, in a total volume of 0.5 mL, 100 mM KCl, 20 mM HEPES-KOH (pH 7.0), 10 mM $MgCl_2$, 50 μM $CaCl_2$, 90 μM EGTA and 0.2 μCi of $^{45}Ca^{2+}$. Following the addition of 2.5 mM ATP, samples (50 μL) were removed at the indicated times and rapidly vacuum-filtered on pre-wetted 0.2 μM nitrocellulose filters (Millipore) and washed with two 1-mL aliquots of 100 mM KCl/10 mM HEPES-KOH (pH 7.0). $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation. The total specific activity of each incubation mixture was determined by counting the radioactivity of an unfiltered 50-μL sample, which was corrected subsequently for non-specific Ca^{2+} binding by filtering a 50-μL sample in the absence of ATP. For *in vitro* $^{45}Ca^{2+}$ accumulation assays, microsomes were prepared from untreated rats. Ciprofibrate (150 μM) and 0.1% DMSO vehicle were either pre-incubated with rat liver microsomes for 5 min or ciprofibrate (150 μM) was added 7 min after the initiation of the reaction.

Western blot analysis of ER Ca^{2+}-ATPase. Immunoblots were performed on microsomal protein preparations separated by sodium dodecyl sulfate (SDS) 7.5% polyacrylamide gel electrophoresis according to Laemmli [26]. Following electrophoresis, gels were transferred to polyvinyldiene difluoride (PVDF) Immobolin membranes in transfer buffer containing 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 10% methanol and adjusted to pH 11 with NaOH. After transfer, the Immobolin membranes were blocked with 0.05% (w/v) Tween-20 in phosphate-buffered saline (0.05% Tween-20/PBS) for 1 hr at room temperature, and then incubated with Ab C4 at a 1:500 dilution of the serum in 0.05% Tween-20/PBS for 1 hr at room temperature [19]. After washing three times for 5 min in 0.05% Tween-20/PBS, membranes were incubated with a rabbit IgG biotinylated secondary antibody in 0.05% Tween-20/PBS for approximately 1 hr at room temperature. Following incubation with the secondary antibody, blots were again washed three times in 0.05% Tween-20/PBS and incubated for 30–45 min at room temperature with an avidin-horseradish peroxidase conjugate antibody. Blots were then washed in PBS three times for 5 min and developed using 0.02% hydrogen peroxide and 4-chloro-1-naphthol as the substrate. Densitometric analysis of Western immunoblots were analyzed using an LKB Ultroscan XL Enhanced laser densitometer.

Measurement of hepatocyte $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} probe indo-1. Hepatocytes were prepared by a two-stage collagenase digestion through livers obtained from fed male rats (200–250 g) as described by Williams *et al.* [27] with minor modifications. Freshly isolated hepatocytes were preincubated at 2.0×10^6 cells/mL in buffer A [modified Krebs–Henseleit buffer containing 10 mM HEPES-KOH, 121 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM $NaHCO_3$, 10 mM D-glucose and 0.2% bovine serum albumin (w/v), pH 7.4] at 37° under an atmosphere of 95% air and 5% CO_2 for 5 min. The cells were then loaded with indo-1-AM (5 μM) for 30 min, after which the cells were washed twice by spinning down at 50 *g* for 2 min and resuspended in fresh buffer A. The indo-1 loaded hepatocytes were then incubated for at least 15 min at 25° to allow for complete de-esterification of indo-1-AM. For measurement of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2 mM $CaCl_2$), hepatocyte suspensions were diluted to 1.0×10^6 cells/mL in buffer A and preincubated at 37° for approximately 5 min prior to the addition of vasopressin or ciprofibrate. For experiments carried out in the absence of extracellular Ca^{2+}, cells (1.0×10^6 cells/mL) were preincubated in buffer A at 37° for 5 min followed by the addition of 1 mM EGTA. After a stable baseline had been achieved cells were then challenged with ciprofibrate. Fluorescence was subsequently monitored at 37° using a Hitachi F-2000 spectrofluorimeter with monochromator settings at 355 nm (excitation) and 405 and 485 nm (emission) detection. $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* [28], using 250 nM as the apparent dissociation constant for Ca^{2+} and indo-1. The calibration values of maximum (F_{max}) and minimum (F_{min}) fluorescence were obtained by permeabilization of the hepatocytes with 0.1% Triton X-100 followed by the addition of 10 μL EGTA (0.25 M in 2.0 M Tris–HCl, pH 8 to 8.5) per mL.

Table 1. Increase in liver to body weight ratio for control and ciprofibrate-exposed male F344 rats

Time (hr)	Control (g liver/100 g body weight)	Ciprofibrate
24	4.58 ± 0.11	5.11 ± 0.12*
48	4.50 ± 0.14	5.83 ± 0.21*
72	4.87 ± 0.25	6.60 ± 0.17*

Male F344 rats were dosed orally with corn oil as control or ciprofibrate (200 mg/kg) and killed at the time-points shown. Data are the means ± SEM for four to six animals at each time-point.

* Statistically significant ($P < 0.01$) compared to controls (Student's *t*-test).

RESULTS

In vivo *time course of ciprofibrate on liver microsomal Ca^{2+}-ATPase activity.* A single oral dose of 200 mg/kg ciprofibrate produced a rapid and substantial increase in the liver to body weight ratio (Table 1), which has also been demonstrated by others [1–3] and is indicative of the acute proliferative response of ciprofibrate in the liver. This response was significant after 24 hr ($P < 0.01$) and animals

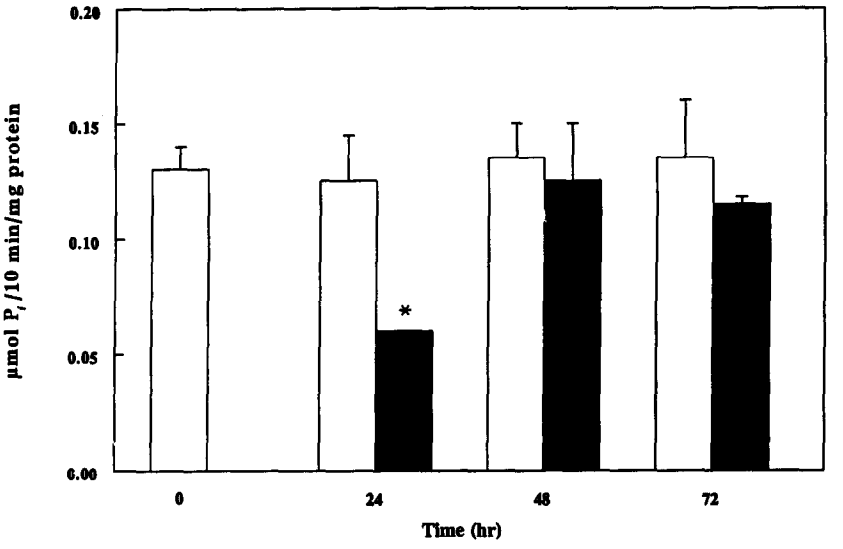

Fig. 1. Ca^{2+}-ATPase activity for control (□) and ciprofibrate-exposed (■) (200 mg/kg) rats. At the time-points indicated, male F344 rats were killed, microsomal fractions were prepared, and Ca^{2+}-ATPase activity was measured immediately as described in Materials and Methods. Data are representative of the means ± SEM of eight separate experiments at 0 hr and four separate experiments at 24, 48 and 72 hr. Data without error bars represents SEM < 5%. Key: (*) statistically significant ($P < 0.05$) compared to respective control (Student's *t*-test).

continued to increase in liver to body weight ratio at all subsequent time points. Measurement of Ca^{2+}-ATPase activity at 24, 48 and 72 hr in control and ciprofibrate-exposed rats was carried out immediately after livers were removed and microsomes were prepared. This was done to ensure that optimal Ca^{2+}-ATPase activity would be measured since freeze–thawing the enzyme tended to reduce activity. Figure 1 shows the results of the time course for ciprofibrate-exposed (200 mg/kg) rats between 24 and 72 hr post-exposure. Ciprofibrate-exposed rats exhibited a significantly decreased microsomal Ca^{2+}-ATPase activity to 48% of control activity at 24 hr post-exposure. Analysis of time points at 48 and 72 hr post-exposure revealed that the Ca^{2+}-ATPase activity of ciprofibrate-exposed rats was restored to levels comparable to those of controls. Ca^{2+}-ATPase activity was also studied 12 hr after ciprofibrate exposure (200 mg/kg), but no differences in Ca^{2+}-ATPase activity between control and ciprofibrate-exposed animals were observed at this time-point (data not shown).

The microsomal fractions prepared in these experiments were enriched with a microsomal marker, glucose-6-phosphatase, to approximately 12-fold over the plasma membrane marker 5′-nucleotidase, thus establishing the purity of these fractions (Fig. 2). The decrease in Ca^{2+}-ATPase activity at 24 hr was selective and not a function of non-specific enzymatic inhibition since the activity of glucose-6-phosphatase remained constant throughout all time-points examined after ciprofibrate exposure. Similarly, 5′-nucleotidase activities measured in microsomal preparations obtained from control and ciprofibrate-exposed (200 mg/kg) animals revealed no significant differences at 24 or 48 hr post-exposure, the former being when Ca^{2+}-ATPase activity was maximally depressed. However, by 72 hr 5′-nucleotidase activity was decreased significantly ($P < 0.05$).

Dose-dependent effect of ciprofibrate on liver microsomal Ca^{2+} *accumulation.* To further investigate the effects of ciprofibrate on Ca^{2+}-ATPase activity at 24 hr, a $^{45}Ca^{2+}$ accumulation assay was employed. ATP-driven accumulation of Ca^{2+} into freshly prepared liver microsomes offered a more sensitive analysis of Ca^{2+}-ATPase activity. In addition, measurement of ATP hydrolysis did not definitively indicate if active Ca^{2+} accumulation in ciprofibrate-exposed rat liver microsomes was also compromised. Accordingly, male F344 rats were exposed to ciprofibrate by gavage at doses of 25, 100 and 200 mg/kg and killed 24 hr later. ATP-driven $^{45}Ca^{2+}$ accumulation was measured in liver microsomes immediately after preparation following removal of livers. Figure 3 shows the dose-dependent kinetics of Ca^{2+} accumulation in rat liver microsomes prepared from control and ciprofibrate-exposed rats 24 hr post-treatment. Ca^{2+} accumulation into liver microsomes after a 10-min incubation (equivalent incubation period used in Ca^{2+}-ATPase assays) demonstrated that rats treated with ciprofibrate at 100 and 200 mg/kg had significantly depressed Ca^{2+} accumulation kinetics relative to control ($P < 0.01$, control animals had comparable Ca^{2+} accumulation kinetics as compared to untreated animals, c.f. Fig. 6). At 25 mg/kg an appreciable reduction of Ca^{2+} accumulating capacity was also observed. These results support our initial findings that ciprofibrate reduced Ca^{2+}-ATPase activity after 24 hr as measured

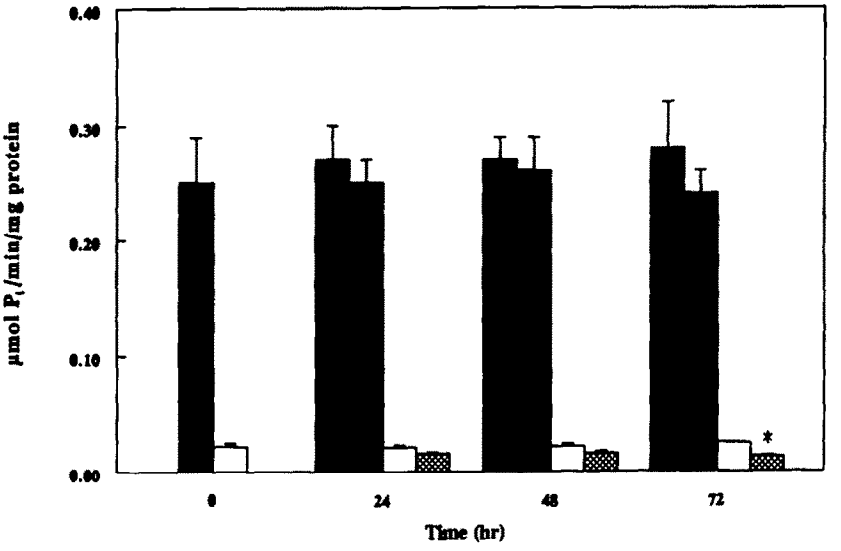

Fig. 2. Activities of glucose-6-phosphatase and 5′-nucleotidase in microsomal fractions obtained from control and ciprofibrate-exposed (200 mg/kg) F344 rats. Values from glucose-6-phosphatase control (■) and ciprofibrate-exposed (▨), and 5′-nucleotidase control (□) and ciprofibrate-exposed (■) rats are the means ± SEM of four to six animals at each time-point. Data without error bars represent SEM < 5%. Key: (*) statistically significant ($P < 0.05$) compared to controls (Student's *t*-test).

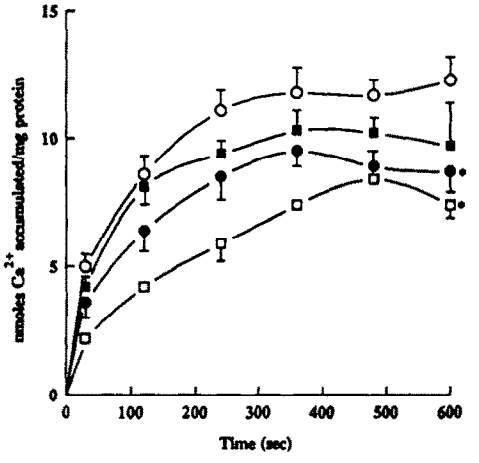

Fig. 3. $^{45}Ca^{2+}$-accumulation kinetics for control (○) and ciprofibrate-treated animals at 25 (■), 100 (●) and 200 (□) mg/kg. Following sacrifice 24 hr after treatment, liver microsomes were immediately prepared and $^{45}Ca^{2+}$ accumulation assays subsequently carried out as described in Materials and Methods. The control represents the mean ± SEM of eight ainimals. Values for ciprofibrate-exposed animals represent means ± SEM for three, five and four animals treated with ciprofibrate at 25, 100 and 200 mg/kg, respectively. For points without error bars SEM < 5%. Key: (*) statistically significant ($P < 0.01$) after a 10-min reaction (corresponding to the incubation period for Ca^{2+}-ATPase assays) as compared to control after 10 min.

using the hydrolysis of ATP and serves to demonstrate that both ATPase and Ca^{2+} accumulating capacities are compromised by ciprofibrate exposure *in vivo*.

Western analysis of liver microsomal Ca^{2+}-*ATPase protein.* Ca^{2+}-ATPase protein content was determined by analysis of Western immunoblots in order to ascertain whether reduced Ca^{2+}-ATPase activity occurring maximally at 24 hr could be attributed to either loss of Ca^{2+}-ATPase protein in microsomal homogenates or to reduced protein content *in vivo*. Figure 4A shows that the Ab C4 cross-reacted with a polypeptide of an apparent molecular mass of 106 kDa. The 106 kDa Ca^{2+}-ATPase protein corresponds closely to that identifed in rabbit cardiac SR which exhibits an apparent molecular mass of 110 kDa [19], and is essentially identical to the recently purified rat liver ER Ca^{2+}-ATPase (107 kDa) [29]. It is important to note that the appearance of an additional band (≈ 105 kDa) closely associated with the 106 kDa Ca^{2+}-ATPase protein is not a distinct polypeptide. The ER Ca^{2+}-ATPase protein exists as several isozymes, ranging from 100 to 110 kDa; this additional band recognized by Ab C4 is most likely to be an associated Ca^{2+}-ATPase isozyme.*

Figure 4B shows a Western immunoblot using Ab C4 on microsomal protein homogenates prepared from control and ciprofibrate-exposed (200 mg/kg) animals 24 hr post-treatment (these microsomal fractions were from the same animals used to obtain the data in Fig. 1). Densitometric analysis of these immunoblots showed that when either 5 or 10 µg of microsomal protein homogenates were analyzed,

* Dr. Jonathan Lytton, personal communication, cited with permission.

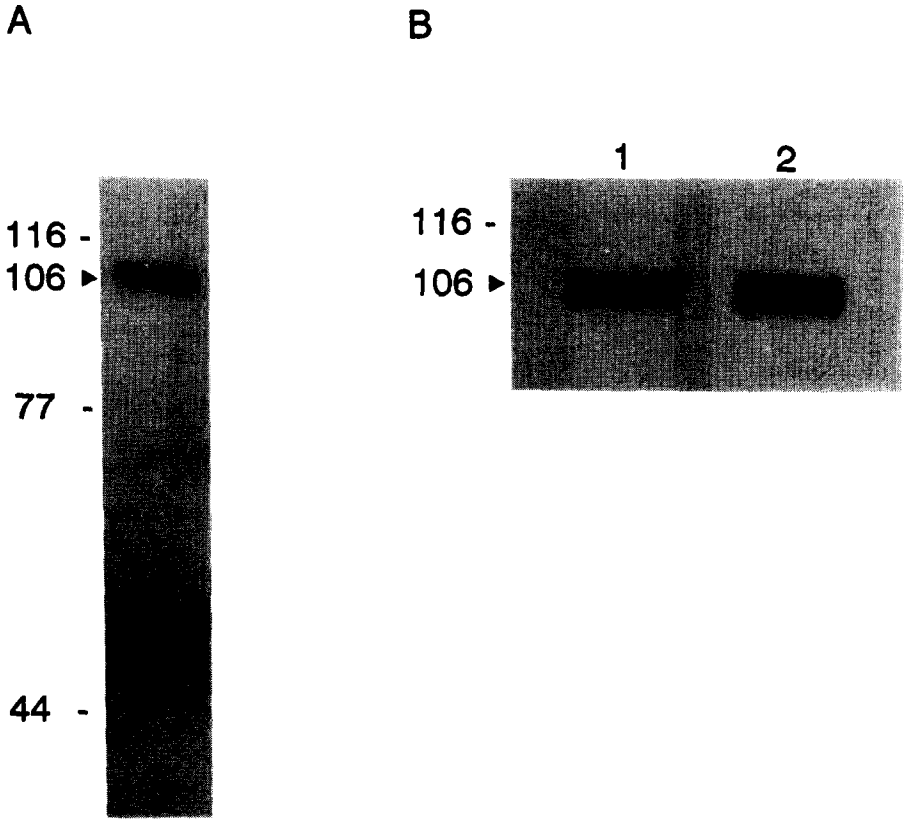

Fig. 4. Western immunoblots of rat liver microsomes using the rabbit cardiac SR Ca^{2+}-ATPase Ab C4. Liver microsomal protein homogenates (5 μg) were separated on a 7.5% SDS polyacrylamide gel, transferred to Immobolin membranes, and probed with Ab C4 at a 1:500 dilution. (A) Shows expression of the 106 kDa ER Ca^{2+}-ATPase protein from untreated rat liver microsomes. (B) Analysis of liver microsomal samples (5 μg) from 24 hr control (lane 1) and ciprofibrate-exposed (200 mg/kg, lane 2) rats with Ab C4. Approximate sizes of the prestained standards (Bio-Rad) in kilodaltons (kDa) are shown on the left.

ciprofibrate-exposed rats exhibited a moderate increase of approximately 20% Ca^{2+}-ATPase protein content relative to control (data not shown). The observation that ciprofibrate-exposed rat liver microsomal fractions do not contain less Ca^{2+}-ATPase protein as compared to controls excludes the possibility that the protein was lost during microsomal preparation. Furthermore, reduced Ca^{2+}-ATPase activity occurring 24 hr post-exposure was not due to diminished protein content *in vivo*; therefore, it is reasonable to suggest a functional mechanism of ciprofibrate-induced Ca^{2+}-ATPase inhibition.

In vitro *effect of ciprofibrate on rat liver microsomal* Ca^{2+}*-ATPase activity*. Using freshly prepared liver microsomes from untreated rats, ciprofibrate produced a concentration-dependent inhibition of Ca^{2+}-ATPase activity *in vitro*, with an apparent IC_{50} of approximately 170 μM (Fig. 5). Addition of the specific Ca^{2+}-ATPase inhibitor tBuHQ which does not affect either plasma membrane or mitochondrial Ca^{2+}-ATPase efflux or sequestration completely abolished Ca^{2+}-ATPase activity at 10 μM as previously reported (Fig. 5, inset) [14, 24]. This demonstrates that minimal plasma membrane or mitochondrial Ca^{2+}-ATPase contamination exists in these fractions, and that the ER Ca^{2+}-ATPase is the predominant component of Ca^{2+}-induced ATP hydrolysis as measured in these fractions. Pre-incubation (5 min) of ciprofibrate (150 μM) with liver microsomes at 37° or addition 7 min after initiation of the reaction also reduced ATP-driven Ca^{2+} accumulation into liver microsomes prepared from untreated rats (Fig. 6).

Ciprofibrate-induced mobilization of hepatocyte $[Ca^{2+}]_i$. Isolated rat hepatocytes displayed a rapid elevation in $[Ca^{2+}]_i$ when treated with vasopressin (50 nM) (Fig. 7A); this transient increase in $[Ca^{2+}]_i$ is typical of agonist-mediated $[Ca^{2+}]_i$ responses which operate via inositol-1,4,5-trisphosphate metabolism [30, 31]. Addition of ciprofibrate (200 μM) to isolated rat hepatocytes produced a slower rise in $[Ca^{2+}]_i$ as compared to the vasopressin response (Fig. 7B), although rapid increases in $[Ca^{2+}]_i$ were observed occasionally. The increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2 mM $CaCl_2$) was sustained, exhibiting an increase of approximately 126 ± 53 nM $[Ca^{2+}]_i$ (mean ± SD, N = 6). The observed increase

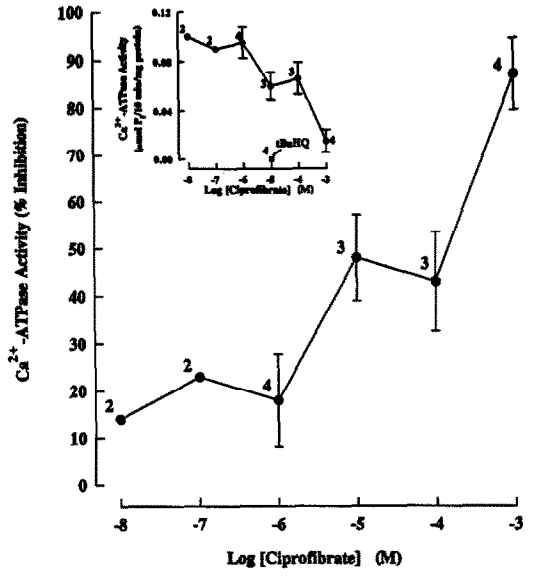

Fig. 5. Concentration-dependent inhibition of ciprofibrate on ER Ca^{2+}ATPase activity. Rat liver microsomes from untreated F344 rats were prepared and incubated with ciprofibrate in the presence (50 μM Ca^{2+}) and absence (1 mM EGTA) of Ca^{2+} at the concentrations shown for 5 min at 37°. The reaction was then initiated with 1 mM ATP and stopped after 10 min with the release of P_i determined by the method of Fiske and SubbaRow [22]. Data are means ± SEM with the number of experiments indicated for Ca^{2+}-ATPase activity (% inhibition) and actual Ca^{2+}-ATPase activity (μmol P_i/10 min/mg protein) (inset). Also represented in the inset is the Ca^{2+}-ATPase activity after incubating microsomes for 5 min with the ER Ca^{2+}-ATPase inhibitor, tBuHQ, which abolished ER Ca^{2+}-ATPase activity.

in $[Ca^{2+}]_i$ was also concentration-dependent, as 100 μM ciprofibrate increased $[Ca^{2+}]_i$ by 58 ± 20 nM (mean ± SD, N = 6). Removal of extracellular Ca^{2+} by the addition of 1 mM EGTA produced a transient increase in hepatocyte $[Ca^{2+}]_i$, although the magnitude of this $[Ca^{2+}]_i$ increase was diminished (Fig. 7C). The structural analogues of ciprofibrate, nafenopin and clofibrate, were also observed to increase hepatocyte $[Ca^{2+}]_i$ at similar concentrations (data not shown).

DISCUSSION

We have shown that following a single dose of ciprofibrate to male F344 rats liver ER Ca^{2+}-ATPase activity was reduced to 48% of control 24 hr after exposure. These results demonstrate that ciprofibrate alters Ca^{2+} homeostasis during the early stages of exposure.

The nature of $[Ca^{2+}]_i$ oscillations is a complex process involving Ca^{2+} itself, $Ins(1,4,5)P_3$ and other Ca^{2+}-dependent transport systems [30–32]. These Ca^{2+} pools can be mobilized by a plethora of polypeptide hormones and growth factors which operate via liberation of $Ins(1,4,5)P_3$ by phospholipase C [31]. The evoked elevation of $[Ca^{2+}]_i$ is essential for activation of a number of critical Ca^{2+}-dependent processes which are involved in cell cycle control and mitogenesis [11, 12]. It is of interest, therefore, to note that changes in Ca^{2+}-ATPase activity occurred prior to the onset of peak DNA synthesis as observed with ciprofibrate and other PPAs [1–3]. The apparent restoration of Ca^{2+}-ATPase activity by 48 hr is unclear, and cannot be explained simply on the basis of elimination of ciprofibrate by the liver. Studies on the pharmacokinetics of ciprofibrate metabolism report that the elimination of ciprofibrate from rats,

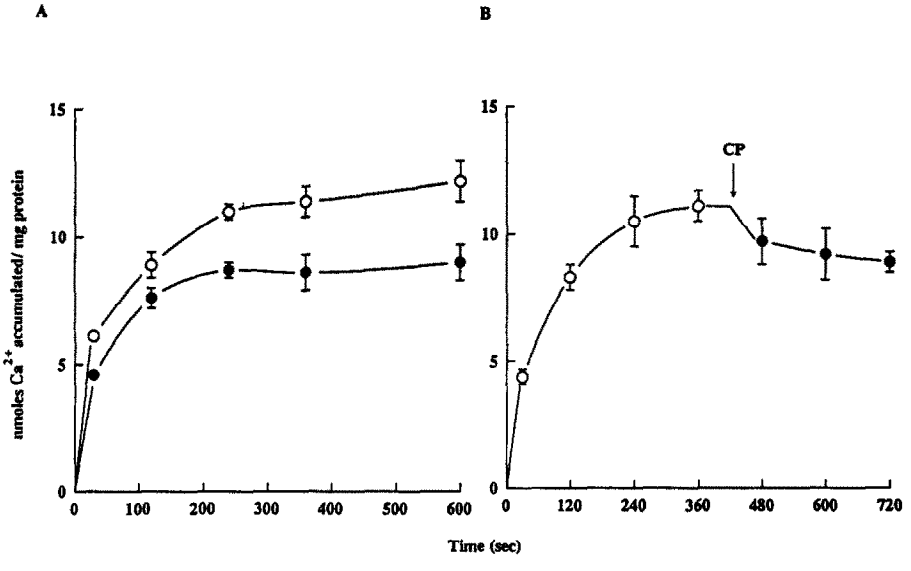

Fig. 6. *In vitro* effect of ciprofibrate (CP) on ATP-driven Ca^{2+} accumulation into liver microsomes prepared from untreated rats. CP (150 μM; ●) or 0.1% DMSO control (○) was either (A) preincubated with microsomal protein for 5 min at 37° prior to the initiation of the reaction or, (B) added 7 min after the initiation of the reaction. Data are the means ± SEM of five and four separate experiments for (A) and (B), respectively. Points without error bars represents SEM < 5%.

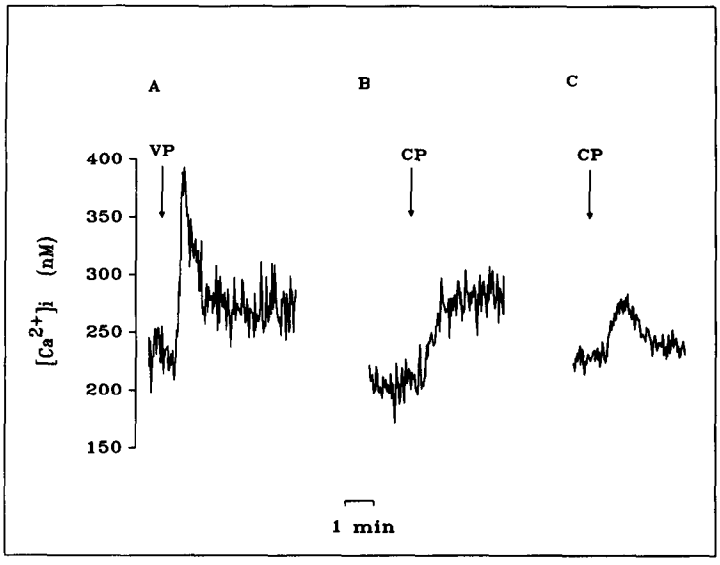

Fig. 7. $[Ca^{2+}]_i$ increase induced by ciprofibrate in isolated rat hepatocytes. $[Ca^{2+}]_i$ was monitored in freshly isolated rat hepatocytes using the fluorescent Ca^{2+} indicator indo-1, as described in Materials and Methods. Key: (A) 50 nM vasopressin (VP); (B) effect of 200 μM ciprofibrate (CP) on hepatocyte $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2.0 mM $CaCl_2$) and (C) in the absence of extracellular Ca^{2+} (1 mM EGTA). These data are representative of at least three separate experiments.

monkeys and humans is extremely slow [33] and is unusually prolonged in the rat which exhibits an elimination half-life of 3–4 days [34, 35]. In addition, studies using whole body autoradiography with [^{14}C]-ciprofibrate in the mouse show that ciprofibrate can be detected in the liver after 4 hr and is still detectable in the liver 9 days later [36]. The nature of this early and transient response in ER Ca^{2+}-ATPase activity *in vivo* remains to be elucidated.

Active Ca^{2+} accumulation into rat liver microsomes was also observed to be compromised in a dose-dependent manner 24 hr after ciprofibrate exposure. Although the kinetics of Ca^{2+} accumulation in ciprofibrate-exposed rats at 25 mg/kg were not statistically significant after 10 min it is important to appreciate that owing to the dynamics of $[Ca^{2+}]_i$ homeostasis large reductions in Ca^{2+}-ATPase activity may not necessarily have to be attained in order to perturb oscillatory $[Ca^{2+}]_i$ fluxes [30, 32]. This is further supported by the fact that the Ca^{2+}-ATPase has a high affinity for Ca^{2+} ($K_m = 0.2\ \mu M$) [13].

To assess non-specific enzyme effects of ciprofibrate a microsomal marker, glucose-6-phosphatase, and a plasma membrane marker, 5′-nucleotidase, were measured. Glucose-6-phosphatase activities in microsomal fractions did not differ between control and ciprofibrate-exposed rats at any of the time-points after ciprofibrate exposure. This observation indicates that the decrease in Ca^{2+}-ATPase activity occurring at 24 hr was not due to non-specific ATPase or other non-specific enzymatic inactivation. Furthermore, the relatively constant level of glucose-6-phosphatase in these microsomal fractions following ciprofibrate exposure indicates that ER redistribution was negligible, eliminating the possibility that reduced Ca^{2+}-ATPase activity was a function of ciprofibrate-induced ER redistribution *in vivo*. 5′-Nucleotidase activity was not affected by ciprofibrate exposure at 24 hr when the inhibition of the Ca^{2+}-ATPase was evident. At 72 hr, however, the plasma membrane enzyme was reduced significantly in activity. Interestingly, alterations in plasma membrane enzymes have been observed previously with the PPAs, clofibrate and nafenopin, which inhibit γ-glutamyltranspeptidase and induce alkaline phosphatase [7]. We have observed that 5′-nucleotidase as measured in microsomal fractions was decreased significantly 72 hr post-ciprofibrate exposure, suggesting that the activity of 5′-nucleotidase residing on the plasma membrane may also be reduced significantly. These observations could constitute a general phenomenon of PPA-induced plasma membrane alterations; the etiology of these changes, however, requires further investigation.

To verify that the reduced ATPase and Ca^{2+} sequestering capacity of the Ca^{2+}-ATPase was not attributed to either a loss of protein during preparation or reduced protein content *in vivo*, we analyzed Ca^{2+}-ATPase protein content using a polyclonal antibody. The polyclonal Ab C4 generated from the rabbit SR Ca^{2+}-ATPase recognized a polypeptide of approximately 106 kDa in isolated rat liver microsomal preparations. Densitometric analysis of Western immunoblots obtained from control and ciprofibrate-exposed rats revealed that ciprofibrate-exposed rats contained a marginally higher proportion of Ca^{2+}-ATPase protein content. This observation eliminates the possibility that the reduced Ca^{2+}-ATPase activity observed maximally

at 24 hr is an artifact of microsomal preparation or reduced Ca^{2+}-ATPase protein content *in vivo*. We can suggest from these data that ciprofibrate-induced Ca^{2+}-ATPase inhibition appears most likely to be a functional inhibition of this enzyme system. Increased levels of Ca^{2+}-ATPase protein in ciprofibrate-exposed rats may be accounted for based on observations by Gorgas and Krisans [37] showing that proliferation of both smooth and rough endoplasmic reticulum occurs with gemfibrozil, a PPA structurally related to ciprofibrate. An increase in ER volume could possibly account for the apparent restoration of Ca^{2+}-ATPase levels at the later time-points. It is of great interest to note that the PPA, clofibrate, has been shown to inhibit Ca^{2+}-ATPase activity of the rabbit cardiac SR [38]. Our findings extend this observation and demonstrate that there is close immunologic homology between rat liver ER Ca^{2+}-ATPase and rabbit SR cardiac isozymes, as also reported by Damiani *et al.* [39] for rabbit skeletal SR.

In isolated rat liver microsomes, ciprofibrate produced a concentration-dependent inhibition of the rat liver ER Ca^{2+}-ATPase, exhibiting an IC_{50} of approximately 170 μM. ATP-driven Ca^{2+} accumulation was also impaired by ciprofibrate *in vitro*, which further supports the interpretation that ciprofibrate inhibits the Ca^{2+}-ATPase activity through a functional alteration of the protein.

To ascertain whether the reduced Ca^{2+}-ATPase activity could lead to an elevation of $[Ca^{2+}]_i$, we investigated the effects of ciprofibrate on hepatocyte $[Ca^{2+}]_i$. Ciprofibrate was found to increase $[Ca^{2+}]_i$ in isolated rat hepatocytes. This increase was concentration-dependent and occurred in the absence of extracellular Ca^{2+}. These results indicate that ciprofibrate mobilizes intracellular Ca^{2+} stores; however, in the absence of extracellular Ca^{2+} the magnitude of the Ca^{2+} signal was reduced suggesting that influx of extracellular Ca^{2+} also plays an important role in the elevation of hepatocyte $[Ca^{2+}]_i$ as induced by ciprofibrate. It is conceivable that ciprofibrate may also alter plasma membrane Ca^{2+} fluxes. Indeed, indirect evidence for ciprofibrate-induced alterations in Ca^{2+} fluxes at the plasma membrane level has been provided by work showing that activation of peroxisomal enzymes by the PPA clofibrate, a structural analogue of ciprofibrate, can be inhibited by Ca^{2+} antagonists such as nicardipine, nifedipine and diltiazem [40]. Ciprofibrate may therefore alter hepatic Ca^{2+} homeostasis by disturbing Ca^{2+} regulation at both the plasma membrane and ER level.

The mechanistic etiology of ciprofibrate-induced Ca^{2+}-ATPase inhibition at this point requires further investigation. Some insight into possible operating mechanisms has been obtained recently in preliminary experiments through use of a thiol immunoblot assay [41]. We found that incubation of rat liver microsomes with ciprofibrate results in a loss of free thiol groups on the ER Ca^{2+}-ATPase, which can be subsequently protected by co-incubation of ciprofibrate with reduced glutathione.* These data suggest that ciprofibrate may inhibit the ER Ca^{2+}-ATPase via an oxidant-induced mechanism. This is supported by other observations that the ER Ca^{2+}-ATPase is susceptible to oxidative damage and inhibition of the enzyme can be achieved using agents that induce oxidative stress and lipid peroxidation [42–46]. However, the rapidity with which active Ca^{2+} accumulation into liver microsomal fractions was reduced *in vitro*, accompanied by the relatively rapid elevation of $[Ca^{2+}]_i$, implicates alternative mechanisms, possibly involving direct interactions between ciprofibrate and free thiol groups on the ER Ca^{2+}-ATPase.

Our finding that ciprofibrate increases hepatocyte $[Ca^{2+}]_i$ corroborates the report of Ochsner *et al.* [17] who showed that the PPAs nafenopin, ciprofibrate and clofibrate all increase $[Ca^{2+}]_i$. In addition, their data show that nafenopin increases $[Ca^{2+}]_i$ independently of inositol phosphate metabolism. These results indicate that nafenopin-induced $[Ca^{2+}]_i$ increases did not occur as a result of $Ins(1,4,5)P_3$ interactions on the ER. These data support our findings which implicate a PPA-induced inhibition of ER Ca^{2+}-ATPase as a mechanism of hepatic $[Ca^{2+}]_i$ mobilization. Nonetheless, alternative sources of ciprofibrate-induced $[Ca^{2+}]_i$ increases must also be considered, such as alteration of plasma membrane Ca^{2+} fluxes which may also contribute to an elevation of $[Ca^{2+}]_i$. Despite these other possible mechanisms, it is clear that ciprofibrate alters hepatic Ca^{2+} homeostasis. This environment may serve to augment Ca^{2+}-dependent processes, and may explain observations that nafenopin potentiates EGF-induced hepatic DNA synthesis in isolated rat hepatocytes [17] and also overcomes the G_1/S block imposed by low extracellular Ca^{2+} [47]. Recently, it has been suggested that the ER Ca^{2+}-ATPase may act as a negative regulator of cell growth, since inhibition of the ER Ca^{2+}-ATPase by thapsigargin causes activation of the protooncogenes *c-fos* and *c-jun* [48]. It is tempting to speculate that ciprofibrate and possibly other PPAs may act by a similar mechanism to induce protooncogene activation. Alterations in hepatic Ca^{2+} homeostasis via ER Ca^{2+}-ATPase inhibition could therefore create a more permissive environment for the activation of multiple Ca^{2+}-dependent processes ultimately leading to a release from growth regulation.

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* Unpublished observations.

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