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CALCIUM AS A PERMISSIVE FACTOR BUT NOT AN INITIATION FACTOR IN DNA SYNTHESIS INDUCTION IN CULTURED RAT HEPATOCYTES BY THE PEROXISOME PROLIFERATOR CIPROFIBRATE

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Abstract—The non-genotoxic hepatocarcinogen and peroxisome proliferating agent, ciprofibrate, is a liver mitogen both *in vivo* and in cultured adult rat hepatocytes, but the mechanisms of its mitogenicity have not been elucidated. We previously observed that ciprofibrate rapidly increased hepatocyte free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), suggesting that this effect may play a role in the initiation of DNA synthesis. In the present study, we have identified a relationship between Ca^{2+} and the stimulation of hepatocyte DNA synthesis by ciprofibrate. Exposure of cultured adult rat hepatocytes to ciprofibrate (200 μM) for 48 hr increased DNA synthesis by approximately 2-fold, and this response was attenuated in a Ca^{2+}-deficient medium and by the Ca^{2+} channel blockers nicardipine and verapamil. To examine the relationship between the stimulation of hepatocyte DNA synthesis and increases in $[Ca^{2+}]_i$ by ciprofibrate, the intracellular Ca^{2+} chelator 5,5′-dimethyl-1,2-bis(2-aminophenoxyethane)-*N,N,N′,N′*-tetraacetic acid (dimethyl-BAPTA) was employed. Pretreatment of hepatocytes with dimethyl-BAPTA blocked ciprofibrate-induced $[Ca^{2+}]_i$ increase, but did not block ciprofibrate-induced hepatocyte DNA synthesis. Dimethyl-BAPTA was only effective in reducing ciprofibrate-induced DNA synthesis when present during the latter 24 hr of a 48-hr culture period. These data suggest that the early mobilization of hepatocyte $[Ca^{2+}]_i$ by ciprofibrate does not play an initiating role in the induction of hepatocyte DNA synthesis but rather may operate as a permissive factor for the entry of ciprofibrate-treated adult rat hepatocytes into S-phase.

Peroxisome proliferating agents (PPAs)‡ have been identified as a structurally diverse group of non-genotoxic rodent hepatocarcinogens [1–3]. Administration of PPAs to rodents causes the proliferation of hepatocytes [4], the mechanisms of which have not yet been elucidated. The aim of this study was to expand upon the observation that the PPA, ciprofibrate, increases hepatocyte $[Ca^{2+}]_i$ [5], which suggested that this response may be important for mitogenesis.

There are a multitude of processes required for the initiation and subsequent progression of cells through the cell cycle [6]. One of these events, the mobilization of free intracellular calcium concentration ($[Ca^{2+}]_i$), is strongly associated with the onset of cell proliferation. The elevation of $[Ca^{2+}]_i$ is a hallmark of the mitogenic response of many growth factors following activation of their cognate receptors. For example, epidermal growth factor [7], platelet-derived growth factor [7], hepatocyte growth factor [8] and transforming growth factor-α [9] all rapidly increase $[Ca^{2+}]_i$ when engaged with their respective receptors. Early rapid increases in $[Ca^{2+}]_i$ have been implicated as the initiating event for mitogenesis [6, 10]. Changes in $[Ca^{2+}]_i$ also play an important role at other stages during the cell cycle; in particular, the progression of cells through S-phase and mitosis has been implicated as Ca^{2+} dependent [11–15]. The observation that nafenopin [16], clofibrate [17], and ciprofibrate [5] also increase hepatocyte $[Ca^{2+}]_i$ suggests that this response may be an important mechanistic event in the initiation of hepatocyte proliferation by the PPA, analogous to that of polypeptide growth factors. To establish whether a relationship may exist between ciprofibrate-induced $[Ca^{2+}]_i$ alterations and the stimulation of hepatocellular DNA synthesis by ciprofibrate, Ca^{2+}-deficient medium, Ca^{2+}-channel blockers and intracellular Ca^{2+} chelators were utilized. Taken together, these experiments reveal that the *early* $[Ca^{2+}]_i$ changes caused by ciprofibrate are not required for the initiation of hepatocellular DNA synthesis. Rather, ciprofibrate-induced alterations

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‡ Abbreviations: PPA, peroxisome proliferating agent; dimethyl-BAPTA, 5,5′-dimethyl-1,2-bis(2-aminophenoxyethane)-*N,N,N′,N′*-tetraacetic acid; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; EGF, epidermal growth factor; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; TCA, trichloroacetic acid; and PBS, phosphate-buffered saline.

in hepatocyte Ca^{2+} homeostasis appear to play an important permissive role allowing the entry of ciprofibrate-treated hepatocytes through the G_1/S boundary.

MATERIALS AND METHODS

Materials. Indo-1-AM and dimethyl-BAPTA-AM [5,5′-dimethyl-1,2-bis(2-aminophenoxyethane)-*N,N,N′,N′*-tetraacetic acid acetomethoxy ester] were obtained from Molecular Probes (Eugene, OR). Ciprofibrate was a gift from Sterling Research Institute, Rensselaer, NY. Epidermal growth factor (EGF), verapamil and nicardipine were purchased from the Sigma Chemical Co. (St. Louis, MO). Collagenase D and [Arg^8]vasopressin were obtained from Boehringer Mannheim (Indianapolis, IN), and [*methyl*-^{3}H]thymidine was from Du Pont NEN Research Products (Boston, MA).

Isolation of adult rat hepatocytes and culture conditions. Cultured rat hepatocytes were isolated using a two-stage collagenase digestion technique through livers obtained from fed male F344 rats as described by Williams *et al.* [18] with minor modifications. Briefly, livers were perfused *in situ* with a Ca^{2+}-free solution [50 mM HEPES-KOH (pH 7.4), 100 mM NaCl, 5.5 mM D-glucose, 5.4 mM KCl, 4.4 mM KH_2PO_4, 3.4 mM Na_2HPO_4, 15 mM $NaHCO_3$, 0.5 mM ethyleneglycolbis(aminoethyl-ether)tetra-acetate (EGTA) and 0.25 mg/mL insulin], followed by a collagenase digestion solution [50 mM HEPES-KOH (pH 7.6), 100 mM NaCl, 5.5 mM D-glucose, 5.4 mM KCl, 15 mM $NaHCO_3$, 6 mM $CaCl_2$, 0.25 mg/mL insulin and 100 U/mL collagenase D]. Hepatocyte viability was assessed by trypan blue exclusion and was consistently in the range of 85–90% viability. For the assessment of replicative DNA synthesis, hepatocytes were cultured at 5×10^4 cells/mL in either complete Williams' Medium E [supplemented with 5% calf serum (Gibco, NY), 2 mM L-glutamine, 10 μg/mL insulin, 4 μg/mL dexamethasone, 100 U/mL penicillin and 100 μg/mL streptomycin] or Ca^{2+}-deficient Williams' Medium E containing either 0.4 or 0.8 mM EGTA. Hepatocyte cultures were maintained for 48 hr at 37° in an atmosphere of 95% air and 5% CO_2. In all experiments ciprofibrate was used at a concentration of 200 μM with dimethyl sulfoxide (DMSO) (0.2%, w/v) as control. Hepatocyte cultures were treated with dimethyl-BAPTA-AM, at 15 μM. Fresh medium and resupplementation with DMSO, ciprofibrate or dimethyl-BAPTA-AM was administered after 24 hr or according to the described protocols.

Assessment of hepatocyte DNA synthesis. Since PPAs do not induce unscheduled DNA synthesis [19], the incorporation of [^{3}H]thymidine is exclusively representative of replicative DNA synthesis (i.e. S-phase). Replicative hepatocyte DNA synthesis was assayed using the incorporation of [^{3}H]thymidine into nuclear DNA [20]. Prior to the termination of each treatment, hepatocytes were pulsed with 4 μCi/mL of [^{3}H]thymidine for 3 hr, after which cultures were washed with cold phosphate-buffered saline (PBS) containing 0.2 mM thymidine and harvested by scraping in Hanks' Buffered Salt Solution. Harvested cells were spun down and resuspended in 0.5 mL of lysis buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$ and 0.65% Nonidet P-40]. Protein concentration of the cell lysate was determined by a modified Bradford method using Coomassie Brilliant Blue G-250 solution (Pierce, Rockford, IL) and bovine serum albumin (Pierce) as standard [21]. The crude nuclear pellet was then precipitated in a microcentrifuge at 800 *g* for 10 min and resuspended in 0.5 mL of 0.25 M sucrose, 50 mM Tris–HCl, 25 mM KCl, 15 mM $MgCl_2$ (pH 8.0) and layered on 0.5 mL of an 0.88 M sucrose, 50 mM Tris–HCl, 25 mM KCl, 15 mM $MgCl_2$ (pH 8.0) high-density gradient. Nuclear isolates were precipitated in a microcentrifuge at 10,000 *g* for 10 min, then treated with 1.0 mL of 0.3 M KOH for approximately 30 min after which the DNA was neutralized with 1 N HCl and precipitated with 10% trichloroacetic acid (TCA). Precipitated DNA was resuspended in 1.5 mL of 5% TCA and hydrolyzed at 80° for 20 min; 1.0-mL aliquot samples of radioactivity were then assessed by liquid scintillation counting.

Measurement of hepatocyte free intracellular Ca^{2+} *concentration.* Hepatocyte $[Ca^{2+}]_i$ was measured with the single excitation, dual emission fluorescent Ca^{2+} probe, indo-1-AM, with monochromator settings at 355 nm (excitation) and 405 and 485 nm (emission) detection. Suspensions of hepatocytes at 2×10^6 cells/mL were preincubated for approximately 15 min in a modified Krebs–Henseleit buffer (Buffer A), containing 10 mM HEPES-KOH, 121 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_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. After preincubation, hepatocytes were loaded with indo-1-AM (5 μM) for 30 min at 37°. Cells were then washed twice by spinning down at 50 *g* for 3 min and resuspended at 1×10^6 cells/mL in fresh Buffer A. Hepatocytes were challenged with either [Arg^8]vasopressin or ciprofibrate, and $[Ca^{2+}]_i$ was determined according to Grynkiewicz *et al.* [22].

Statistical analysis. The data presented in Figs. 2–4 were analyzed using a one-way analysis of variance, followed by Scheffe's test [23] for pairwise comparison of each experimental group with the control. P values are represented as a function of the logarithmic transformation applied to the raw data to stabilize the error variance.

RESULTS

Effect of ciprofibrate on hepatocyte $[Ca^{2+}]_i$. The hormone [Arg^8]vasopressin mobilizes hepatocyte $[Ca^{2+}]_i$ via generation of $Ins(1,4,5)P_3$, and stimulates a transient elevation in hepatocyte $[Ca^{2+}]_i$ [24] as demonstrated in indo-1-loaded rat hepatocytes (Fig. 1A). In contrast, exposure of hepatocytes to ciprofibrate at 100 and 200 μM induced a concentration-dependent and sustained elevation of hepatocyte $[Ca^{2+}]_i$ (Fig. 1), as previously observed [5]. The sustained elevation of $[Ca^{2+}]_i$ induced by ciprofibrate is attributed to reduced Ca^{2+} sequestration as a result of the inhibition of the endoplasmic reticulum (ER) Ca^{2+}-ATPase by ciprofibrate [5, 25]. This is supported by the

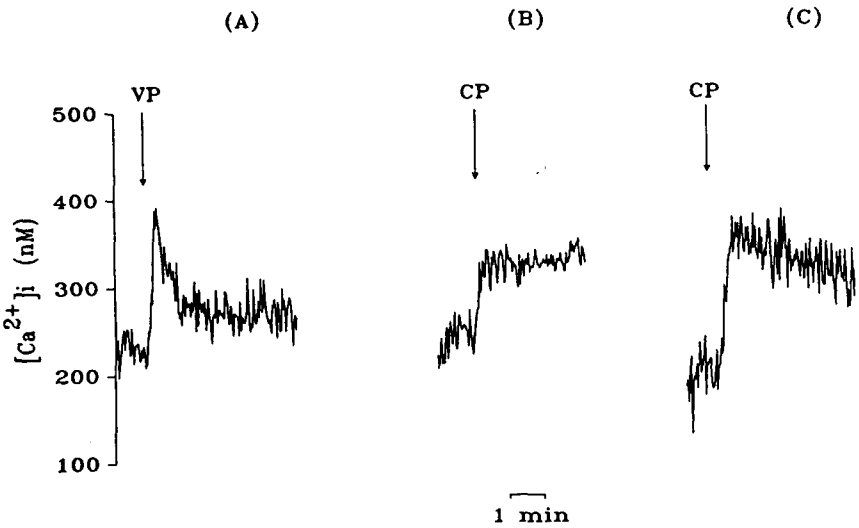

Fig. 1. Effect of ciprofibrate (CP) on hepatocyte $[Ca^{2+}]_i$. Freshly isolated suspensions of rat hepatocytes were loaded with the fluorescent Ca^{2+} probe, indo-1 (5 μM), and $[Ca^{2+}]_i$ was measured in response to (A) 50 nM [Arg^8]vasopressin (VP), (B) 100 μM ciprofibrate and (C) 200 μM ciprofibrate, as described in Materials and Methods.

observation that nafenopin also increases hepatocyte $[Ca^{2+}]_i$, but does so independently of the generation of Ins(1,4,5)P_3 [16]. Thus, ciprofibrate has the ability to produce a sustained elevation of hepatocyte $[Ca^{2+}]_i$ in a manner that is characteristic of other ER CA^{2+}-ATPase inhibitors such as 2,5-di-*tert*-butylbenzohydroquinone [26] and thapsigargin [27].

Dependency upon extracellular Ca^{2+} *for ciprofibrate-induced hepatocellular DNA synthesis.* Stimulation of DNA synthesis in cultured rat hepatocytes by the PPA has been demonstrated by numerous groups [19, 28–30]. Induction of hepatocyte DNA synthesis by ciprofibrate as well as other PPAs was not observed until 48 hr after exposure *in vitro*, at which point DNA synthesis was increased significantly by 1.7- to 2.4-fold (Fig. 2, top panel), in addition, hepatocyte cell number also increased by approximately the same order of magnitude (unpublished observations). The DNA synthetic wave of hepatocytes in culture diminishes after 72 hr in response to PPAs [19, 31], indicating that maximal DNA synthesis occurs after 48 hr *in vitro*. This is consistent with the observation that cultured primary hepatocytes emerge (almost simultaneously) from G_0 and progress through to S-phase within 36–48 hr [32]. In the absence of serum, ciprofibrate also increased hepatocyte DNA synthesis by the same order of magnitude (data not shown), indicating that serum factors play a negligible role in the induction of DNA synthesis by ciprofibrate. In light of these experiments, we proceeded to assess the effects of ciprofibrate on hepatocyte DNA synthesis induction at 48 hr in complete Williams' Medium E.

When hepatocytes were cultured for 48 hr in a Ca^{2+}-deficient medium, maximal ciprofibrate-induced hepatocyte DNA synthesis was attenuated significantly (Fig. 2, bottom panel). These data indicate that ciprofibrate-induced hepatocellular DNA synthesis is dependent upon extracellular Ca^{2+}. Under similar conditions, when hepatocytes were exposed to EGF (100 ng/mL) in a Ca^{2+}-deficient medium, maximal EGF-induced hepatocyte DNA synthesis was attenuated by up to 50% (Fig. 2, inset), as also reported by Armato *et al.* [33]. Thus, maximal hepatocyte DNA synthesis in response to ciprofibrate and EGF is dependent upon extracellular Ca^{2+}.

Influx of extracellular Ca^{2+} into cells occurs through both voltage-operated [34] and receptor-operated Ca^{2+} channels [35]. Therefore, we anticipated that use of the L-type voltage-operated Ca^{2+} channel blockers, nicardipine and verapamil, could potentially block ciprofibrate-induced hepatocyte DNA synthesis, if indeed the influx of extracellular Ca^{2+} plays a significant role in this process. Treatment of cultured rat hepatocytes with nicardipine and verapamil at 1, 10 and 25 μM inhibited ciprofibrate-induced hepatocellular DNA synthesis in a concentration-dependent manner (Fig. 3). The concentrations of ciprofibrate, nicardipine, verapamil and all subsequent combinations of culture treatments (see below) were not toxic during the 48-hr culture period as determined by trypan blue dye exclusion (data not shown).

Modulation of ciprofibrate-induced DNA synthesis by the intracellular Ca^{2+} *chelator BAPTA.* As demonstrated in Fig. 1, ciprofibrate elevated hepatocyte $[Ca^{2+}]_i$, which suggested that the *early* alterations in Ca^{2+} homeostasis may potentially be an important component in the initiation of the mitogenic response. To examine this, hepatocytes were pretreated with an intracellular Ca^{2+} chelator to determine whether the *early* ciprofibrate-induced $[Ca^{2+}]_i$ alterations are important for the stimulation of hepatocyte DNA synthesis by ciprofibrate. At

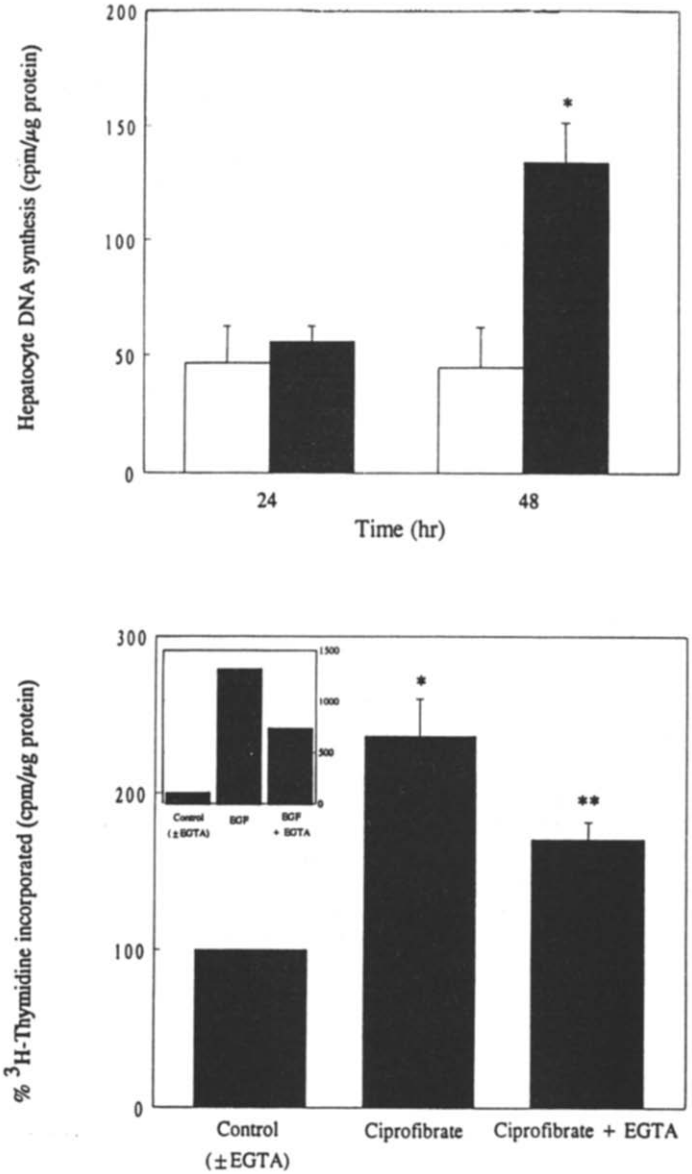

Fig. 2. (Top panel) Induction of hepatocyte DNA synthesis by ciprofibrate. Adult rat hepatocytes were cultured in complete Williams' Medium E in the absence (□) or presence of ciprofibrate (200 μM; ■). Hepatocyte DNA synthesis was assessed at the given time points, as described in Materials and Methods. Data represent the means ± SEM of three and fifteen separate experiments at 24 and 48 hr, respectively. Key: (*) statistically significant at $P < 0.05$, as compared with respective control (see Materials and Methods). (Bottom panel) Ciprofibrate-induced hepatocyte DNA synthesis in normal and Ca^{2+}-deficient media. Adult rat hepatocytes were cultured in either complete or Ca^{2+}-deficient Williams' Medium E with ciprofibrate (200 μM) for 48 hr. Data represent means ± SEM of five separate experiments. Key: (*) statistically significant at $P < 0.05$, as compared with control and (**) statistically significant at $P < 0.05$ as compared with ciprofibrate-treated cultures. Inset: hepatocyte DNA synthesis following a 48-hr treatment with 100 ng/mL EGF in the presence of complete or Ca^{2+}-deficient Williams medium E.

concentrations ranging from 10 to 20 μM, BAPTA has been shown to block agonist-induced $[Ca^{2+}]_i$ increases [36] as well as physiologically responsive Ca^{2+}-dependent events such as tyrosine phosphorylation induced by the Ca^{2+} mobilizing hormone angiotensin II and by the ER Ca^{2+}-ATPase inhibitor thapsigargin [37]. In our experiments, treatment of hepatocytes with 15 μM dimethyl-BAPTA (or BAPTA) abolished the increase in $[Ca^{2+}]_i$ induced by ciprofibrate at 200 μM, the maximal concentration used in these experiments (data not shown).

The treatment protocols (1–8) employed in these experiments are depicted in Fig. 4A, and the corresponding effects on hepatocyte DNA synthesis

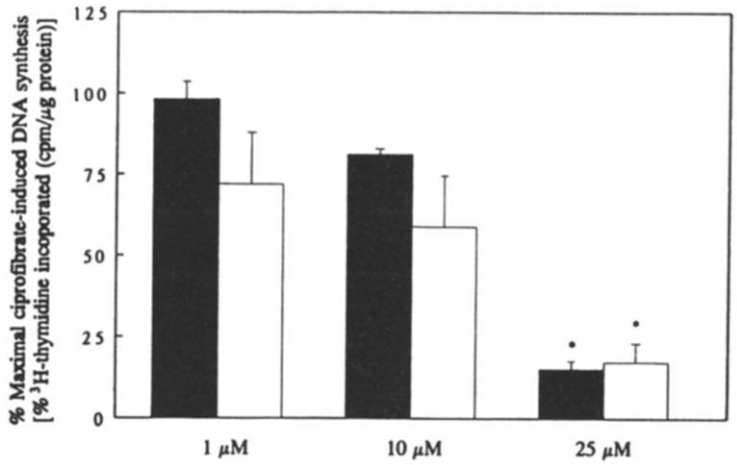

Fig. 3. Effect of the Ca^{2+}-channel blockers nicardipine and verapamil on ciprofibrate-induced hepatocyte DNA synthesis. Cultured rat hepatocytes were exposed to ciprofibrate plus nicardipine (■) or verapamil (□) at the concentrations shown. Hepatocyte DNA synthesis was assessed after 48 hr as described. Data represent means ± SEM of four to five separate experiments. Key: (*) statistically significant at $P < 0.05$ from ciprofibrate-treated control cultures.

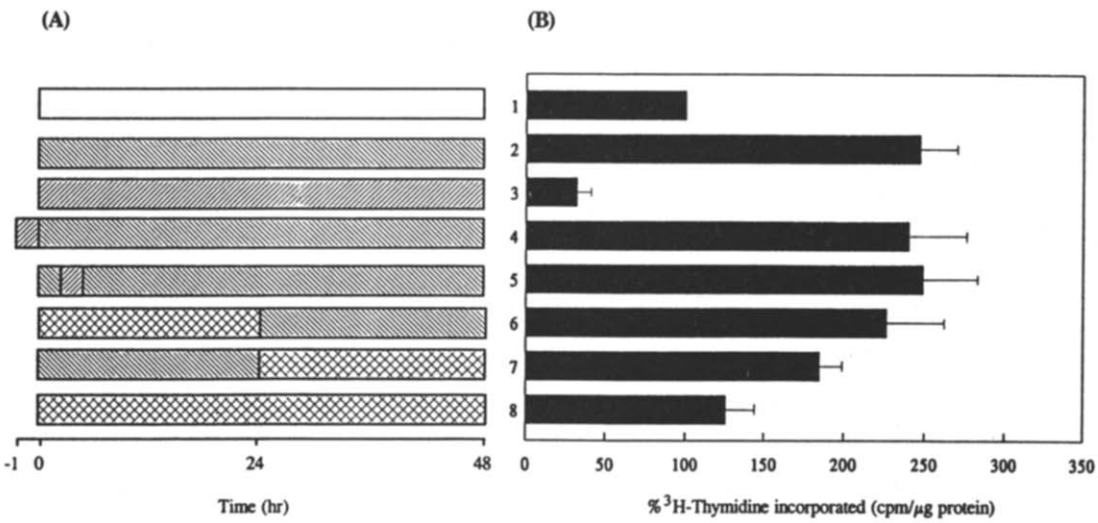

Fig. 4. Modulation of ciprofibrate-induced hepatocellular DNA synthesis by the intracellular Ca^{2+} chelator dimethyl-BAPTA. (A) Cultured rat hepatocytes were treated according to the dosing regimen depicted in bars 1–8. Key: (□) control, (▨) ciprofibrate (200 μM), (▨) dimethyl-BAPTA (15 μM), and (■) ciprofibrate (200 μM) plus dimethyl-BAPTA (15 μM). (B) Forty-eight hours after treatment, hepatocyte DNA synthesis was assessed by the incorporation of [^{3}H]thymidine in response to the corresponding treatment regimens (1–8). Data are representative of three to four separate experiments (means ± SEM). Identical results were also obtained using the parent intracellular Ca^{2+} chelator BAPTA at 15 μM (data not shown).

in response to the various treatments are shown in Fig. 4B. Ciprofibrate typically increased hepatocyte DNA synthesis by approximately 2-fold as observed previously (treatment 2). Continual exposure of cultured hepatocytes to dimethyl-BAPTA for 48 hr reduced basal hepatocyte DNA synthesis (treatment 3). When hepatocytes were loaded with the intracellular Ca^{2+} chelator dimethyl-BAPTA for 1 hr, followed by removal and subsequent exposure with ciprofibrate for 48 hr, no attenuation in ciprofibrate-induced DNA synthesis was observed (treatment 4). Since pretreatment with dimethyl-BAPTA had no significant effect on ciprofibrate-induced hepatocyte DNA synthesis, this indicated that the *early* ciprofibrate effects on $[Ca^{2+}]_i$ were not essential for the initiation of maximal hepatocyte

DNA synthesis. Post-treatment with dimethyl-BAPTA for 1 hr, following 1 hr of ciprofibrate treatment, also produced no significant effect on ciprofibrate-induced hepatocyte DNA synthesis (treatment 5). This particular treatment precludes the involvement of any $[Ca^{2+}]_i$ changes occurring shortly after ciprofibrate exposure. Simultaneous administration of both ciprofibrate and dimethyl-BAPTA for 48 hr to hepatocytes reduced hepatocyte DNA synthesis in response to ciprofibrate (treatment 8). The presence of dimethyl-BAPTA during the first 24-hr period of the 48-hr exposure was ineffective at reducing ciprofibrate-induced DNA synthesis (treatment 6). When however, dimethyl-BAPTA was present during the latter 24 hr (24–48 hr) of the incubation, ciprofibrate-induced hepatocyte DNA synthesis could be reduced (treatment 7). The treatment protocols where dimethyl-BAPTA either was pulsed for 1 hr after ciprofibrate treatment or was present during the first 24 hr only exclude the possibility of non-specific and/or toxic effects of dimethyl-BAPTA on hepatocyte function, since with subsequent removal of dimethyl-BAPTA maximal hepatocyte DNA synthesis was still attained. Although treatment groups 7 and 8 were not statistically significant when compared to the ciprofibrate-exposed cultures, these data represented consistent biological events. This is further supported by the finding that identical results were also obtained with the parent intracellular Ca^{2+} chelator BAPTA (data not shown). These experiments can be interpreted to indicate that the *early* $[Ca^{2+}]_i$ changes are not the determinant signal for the initiation of DNA synthesis by ciprofibrate. Nonetheless, the findings indicate that the $[Ca^{2+}]_i$ changes during the latter stages of ciprofibrate exposure are important for maximal ciprofibrate-induced hepatocyte DNA synthesis.

DISCUSSION

In this study we have examined the role of extracellular Ca^{2+} and $[Ca^{2+}]_i$ in the initiation of DNA synthesis in cultured rat hepatocytes by ciprofibrate. The relationship between *early* $[Ca^{2+}]_i$ changes and the subsequent onset of DNA synthesis is a difficult association to establish with respect to cause and effect based only on the sequence of these two events. Nevertheless, using a combination of several Ca^{2+} modulating protocols that target distinct Ca^{2+} pathways, coupled with a temporal sequence of application, we have identified the window in which alterations in $[Ca^{2+}]_i$ are important for maximal enhancement of ciprofibrate-induced DNA synthesis. These data indicate that the *early* increase in hepatocyte $[Ca^{2+}]_i$ stimulated by ciprofibrate is not the determinant signal for the subsequent initiation of hepatocyte DNA synthesis. We had anticipated that the *early* $[Ca^{2+}]_i$ increases would constitute the initiating event of ciprofibrate-induced DNA synthesis in a manner similar to other growth factors [10]. This, however, does not appear to be the case. Nonetheless, we made other observations with respect to similarities between ciprofibrate-induced hepatocyte DNA synthesis and growth factor-induced DNA synthesis. First, the finding that ciprofibrate-induced DNA synthesis was dependent upon extracellular Ca^{2+} has also been made with EGF [38]. Interestingly, in this same report [38], it was also demonstrated that nafenopin-induced hepatocellular DNA synthesis was independent of extracellular Ca^{2+} in neonatal rat hepatocytes. The reason for this difference may be attributable to the significant dissimilarities between neonatal and adult rat hepatocytes; that neonatal rat hepatocytes possess a higher population of cycling cells is sufficient reason to suspect that nafenopin-induced DNA synthesis was independent of extracellular Ca^{2+}. Second, the Ca^{2+} channel blockers have also been demonstrated to inhibit growth factor-induced cell proliferation in a variety of cell types [10, 39], presumably as a result of inhibiting Ca^{2+} influx. Verapamil at concentrations similar to those used in the present experiments has also been shown to inhibit EGF-induced hepatocyte DNA synthesis [40], corroborating our observation on the dependency of EGF-induced hepatocyte DNA synthesis for extracellular Ca^{2+}. These data provide strong evidence that ciprofibrate-induced DNA synthesis is dependent upon extracellular Ca^{2+} in a manner similar to EGF. Whether there is a Ca^{2+} dependency for mitogenesis induced by other PPAs remains to be investigated. Nevertheless, since other PPAs also mobilize hepatocyte $[Ca^{2+}]_i$ [16, 17], one would predict that a similar requirement for Ca^{2+} for maximal DNA synthesis would also exist.

Ciprofibrate-induced hepatocyte DNA synthesis was also inhibited by nicardipine and verapamil. Interestingly, the Ca^{2+} channel blockers nicardipine, diltiazem and nifedipine have also been reported to inhibit the stimulation of the peroxisomal fatty acyl-CoA oxidizing system by clofibrate [41], suggesting that a Ca^{2+}-dependent mechanism may be operating in PPA-induced peroxisome proliferation. In a follow-up study, again using clofibrate and nicardipine, Itoga *et al.* [42] observed that the initial induction of peroxisomal enzyme activity by clofibrate only became suppressive after 5 days of treatment. This was interpreted to suggest that the induction of peroxisomal enzymes has two phases: first, an initiating event, and second, a progression event. In this context, nicardipine was suggested to act during the latter phases to inhibit peroxisomal enzyme induction. From an operational standpoint, the permissive role of Ca^{2+} in peroxisomal enzyme induction is similar to our observations, in that changes in $[Ca^{2+}]_i$ during the latter periods of hepatocyte culture also appear to play a permissive role in ciprofibrate-induced DNA synthesis. Despite these apparent similarities, a dilemma arises at this juncture; since both hepatocyte DNA synthesis and peroxisomal enzyme induction appear to be inhibited by the Ca^{2+} channel blockers, the ability to dissect these two processes using Ca^{2+} channel blockers is confounded. It is quite conceivable that altered Ca^{2+} homeostasis may act as an important permissive factor for both maximal peroxisomal enzyme induction as well as PPA-induced hepatocellular DNA synthesis. Further studies are obviously required to clarify this situation.

The influx of extracellular Ca^{2+} was found to be an important factor for maximal ciprofibrate-induced

hepatocyte DNA synthesis, but it is necessary to stress that the Ca^{2+} channel blockers have also been reported to inhibit the mobilization of Ca^{2+} from intracellular stores [39, 43]. This may explain the observation that nicardipine and verapamil were more inhibitory on ciprofibrate-induced hepatocyte DNA synthesis than was low extracellular Ca^{2+} alone. This explanation may also be applied to the use of the intracellular Ca^{2+} chelator, dimethyl-BAPTA, which also inhibited ciprofibrate-induced DNA synthesis to a higher degree than low extracellular Ca^{2+} alone (see below). This is consistent with the fact that dimethyl-BAPTA buffers $[Ca^{2+}]_i$ increases, irrespective of the source. These observations also provide evidence that both intracellular and extracellular Ca^{2+} sources contribute to maximal ciprofibrate-induced hepatocellular DNA synthesis.

Application of dimethyl-BAPTA at different time-points in this study allowed us to establish that the *early* $[Ca^{2+}]_i$ changes were not operative in the initiation of hepatocyte DNA synthesis by ciprofibrate. The observation that ciprofibrate-induced hepatocyte DNA synthesis can be attenuated significantly by exposure to dimethyl-BAPTA during the latter 24 hr of culture but not when dimethyl-BAPTA was present during the first 24 hr, suggests that changes in $[Ca^{2+}]_i$ play an important role in the latter stages of ciprofibrate mitogenesis, presumably at the G_1/S boundary. The interpretations from these experiments do not appear to be confounded by non-specific or toxic dimethyl-BAPTA effects. This is based on the observation that the 1-hr post-treatment with dimethyl-BAPTA and the early 24-hr dimethyl-BAPTA treatment did not irreversibly block hepatocyte function since subsequent removal of dimethyl-BAPTA either after a post 1-hr pulse or after 24 hr did not affect maximal ciprofibrate-induced DNA synthesis. The finding that ciprofibrate-induced $[Ca^{2+}]_i$ increases do not appear to play an initiating role for hepatocyte DNA synthesis, but rather may act as a permissive factor, is supported by the observation that maximal PPA-induced DNA synthesis can only be achieved when hepatocytes are treated continually with a PPA [19].

A role for $[Ca^{2+}]_i$ as an initiating factor for DNA synthesis has been demonstrated by Diliberto *et al.* [10] who showed that platelet-derived growth factor-induced DNA synthesis could be suppressed by buffering $[Ca^{2+}]_i$ elevations in BALB/c3T3 fibroblasts by pretreatment with the intracellular Ca^{2+} chelator TMB-8. Conversely, studies on growth factor-mediated mitogenesis by the fibroblast growth factor receptor indicate that increases in $[Ca^{2+}]_i$ produced by the fibroblast growth factor do not participate in the initiation of mitogenesis in L6 myoblasts [44, 45]. Taken together, these observations indicate that both Ca^{2+}-dependent and -independent pathways exist for the initiation of DNA synthesis, and data presented here clearly show that ciprofibrate utilizes a Ca^{2+}-independent pathway for the initiation of DNA synthesis.

Ciprofibrate increases hepatocyte $[Ca^{2+}]_i$ through inhibition of the ER Ca^{2+}-ATPase by altering the thiol integrity of this enzyme [5, 25]. Recently, several studies have addressed the role of ER Ca^{2+}-ATPases in cell proliferation. One group reported that inhibition of the ER Ca^{2+}-ATPase using thapsigargin resulted in the activation of the mitogen responsive transcription factors c-*fos* and c-*jun*, with c-*fos* being activated in a Ca^{2+}-dependent manner [46]. On the other hand, Ghosh *et al.* [47] suggested that emptying of Ca^{2+} pools completely inhibits DNA synthesis and the proliferation of DDT_1MF-2 cells, again using thapsigargin. Lastly, Magnier *et al.* [48] found that following platelet-derived growth factor stimulation of smooth muscle cells, the $SERCA_2$-type Ca^{2+}-ATPase is increased. It is clear that the ER Ca^{2+}-ATPase controls the major Ca^{2+} stores mobilized by polypeptide growth factors. Therefore, its intrinsic activity and regulation must in some way impinge upon the control of cell proliferation. However, no definitive conclusions can be made with regard to whether inhibition of ER Ca^{2+}-ATPase activity is either stimulatory or inhibitory to cell proliferation. We had suggested previously that ciprofibrate-induced increases in $[Ca^{2+}]_i$ could potentially act as an initiating or a permissive factor or both for ciprofibrate mitogenesis [5]. The present studies show that ciprofibrate engages the Ca^{2+} signal transduction pathway which is subsequently required for maximal stimulation of hepatocyte DNA synthesis. Nonetheless, the essential initiating event(s) of ciprofibrate-induced DNA synthesis still remains to be elucidated.

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