TABLE 2. Changes in Mean ΔS_{BC} Values in Rat Couplet Hepatocytes After Addition of 1 μmol/L ZnPP in the Absence and Presence of 1 μmol/L CO in the Medium

Groups	ΔS_{BC} (μm^2)	
	40-min Control Period	40-min ZnPP Treatment
1 μmol/L ZnPP	3.72 ± 0.67	2.44 ± 0.34*
1 μmol/L ZnPP + 1 μmol/L CO	3.57 ± 0.76	3.01 ± 1.14

NOTE. Data were collected from the replayed videotapes over 80 minutes (40 minutes each before and after the ZnPP application), representing means ± SD of measurements from 30 to 40 occasions of BC contractions in four to five different hepatocyte couplets. CO at 1 μmol/L was applied simultaneously with ZnPP.

*$P < .05$ as compared with the control values estimated under the ZnPP-free conditions in the corresponding couplets. Analysis was performed by the Student's t test for paired values.

ZnPP. These results suggest that a decrease in the basal $[Ca^{2+}]_i$ by the intracellular Ca^{2+} chelator can abolish the ZnPP-induced $[Ca^{2+}]_i$ elevation.

We next tried to examine whether elimination of Ca^{2+} in the culture medium could abolish the ZnPP-induced Ca^{2+} responses in the hepatocytes. Because calcium-free culture conditions did not allow us to maintain attachment of isolated hepatocytes on culture dishes, the experiments were performed by using the hepatocyte suspension. Figure 8B illustrates the time history of the fura-2–associated fluorescence (I_{340}/I_{380}) in the cell suspension incubated in the presence or absence of calcium ion. As seen, no difference in the ZnPP-evoked response of $[Ca^{2+}]_i$ was observed between the two groups. These findings suggest that the ZnPP-induced $[Ca^{2+}]_i$ elevation is ascribable to Ca^{2+} mobilization from the intracellular pool, rather than that from the extracellular space.

We further inquired whether administration of ZnPP might influence the intensity of fura-2 fluorescence in hepatocytes, because free Zn^{2+} is known to form a fluorogenic complex with fura-2.[21] For that purpose, effects of pretreatment with TPEN, a reagent that chelates heavy metals such as Zn^{2+} and Mn^{2+} but not Ca^{2+}, were examined. Application of 50 μmol/L TPEN did not alter the baseline fura-2 fluorescence in hepatocyte couplets. When 1 μmol/L ZnPP was then applied, the initial increase in $[Ca^{2+}]_i$ was blunted a little, but the maximum response of $[Ca^{2+}]_i$ was similar to that observed in the TPEN-free couplets (data not shown).

Effects of CO on cGMP Levels. We examined the CO-elicited response of cGMP in the cultured hepatocytes. As seen in Table 3, cGMP contents were not significantly changed by the application of 2 μmol/L CO. Even by increasing the CO concentration to 20 μmol/L, the cGMP contents did not display any significant elevation. On the other hand, 100 μmol/L SNAP elicited approximately an eightfold increase in the cGMP contents, indicating that guanylate cyclase is present in these cells and activated by nitric oxide.[5] The cGMP levels in the hepatocytes were not altered by the application of ZnPP at 1 μmol/L, suggesting that the inhibitory action of this reagent on soluble guanylate cyclase[13] does not contribute to the attenuation of ZnPP-induced activation of BC contraction. Furthermore, application of 1 μmol/L FePP, a substrate of heme oxygenase, also evoked no changes in the cGMP contents. These results suggest that activation of guanylate cyclase is unlikely to account for the mechanism of the inhibitory action of CO on the BC contraction in hepatocyte couplets.

DISCUSSION

Results obtained in this study indicated that CO generated in hepatocytes serves as an endogenous regulator of BC contraction for the following reasons. First, isolated rat hepatocytes were able to produce a significant amount of CO comparable with that determined in the perfused liver preparation.[6] Second, ZnPP, a potent inhibitor of heme oxygenase, which abolished endogenous CO production in the cells, induced an increase in the frequency of BC contractions concurrent with a decrease in the stroke change

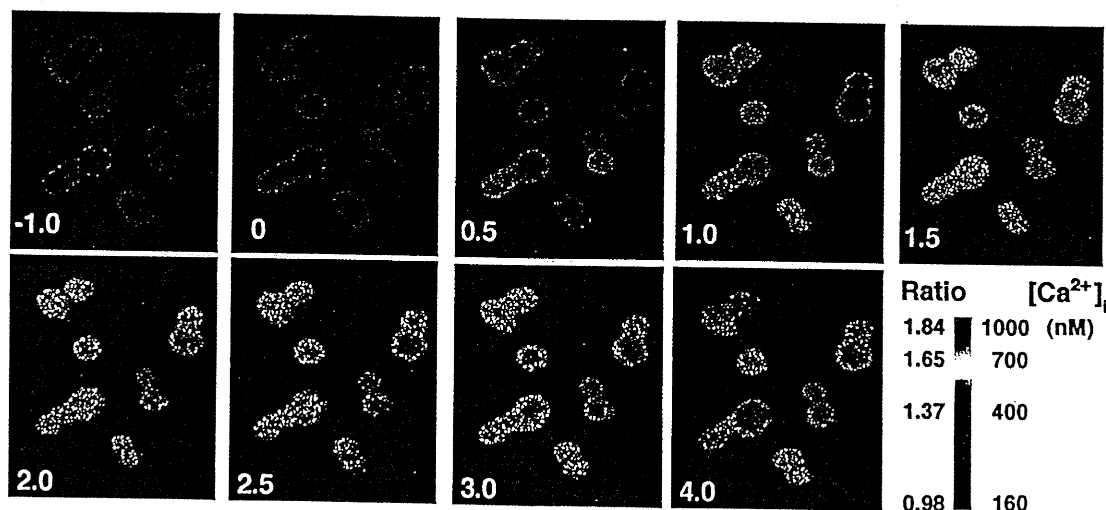

FIG. 7. Representative serial pictures of fura-2–assisted microfluorographs in rat hepatocyte couplets treated with 1 μmol/L ZnPP. *Color bars* indicate calibrated values of intracellular Ca^{2+} concentrations. The ZnPP superfusion was started at time 0. Note the heterogeneous responses in the increase in the calcium concentrations among hepatocyte couplets.

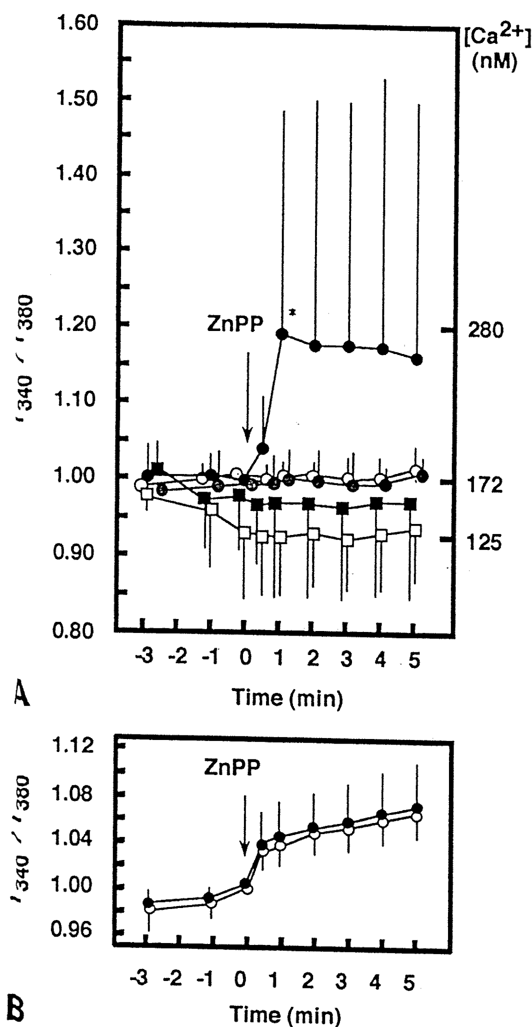

FIG. 8. Time history of the intracellular calcium concentrations in the ZnPP-treated rat hepatocytes. (A) Intracellular calcium concentrations measured in the ZnPP-treated hepatocyte couplets and effects of treatment with 1 μmol/L CO, 1 μmol/L clotrimazole, and 50 μmol/L BAPTA-AM. (○), (●), and (●) denote data collected from the control, ZnPP-treated, and ZnPP- + CO-treated couplets. (■) and (□) indicate those collected from ZnPP-applied couplets pretreated with clotrimazole and BAPTA-AM, respectively. CO, clotrimazole, and BAPTA-AM were applied to the culture medium 3 minutes before the ZnPP administration. Data represent mean ± SD of measurements from 20 to 25 different hepatocyte couplets isolated from four separate liver preparations. Note that the ZnPP treatment induces a significant increase in $[Ca^{2+}]_i$ despite heterogeneity of the response as indicated by large SD values. (B) Effects of elimination of calcium ion in the extracellular medium on the ZnPP-induced increase in the intracellular calcium concentrations. These measurements were performed by fura-2–assisted fluorescence spectrophotometry using the hepatocyte suspension. (○) and (●) illustrate measurements performed in the absence and presence of 1.3 mmol/L $CaCl_2$, respectively, showing no substantial difference between the two groups.

in canalicular areas. Finally, a supplement of CO applied exogenously at 1 μmol/L, a concentration that is comparable with that detected in the venous effluents of the perfused liver,[6] attenuated the ZnPP-induced increase in the frequency of BC contraction. We described previously that endogenously generated CO plays a role in active relaxation of sinusoidal vessels to maintain the low vascular resistance.[6,27] Furthermore, our recent study has demonstrated that HO-2, the constitutive heme oxygenase isozyme, in hepatocytes constitutes a major cellular component for endogenous CO generation that occurs in the extravascular space in the liver.[24] Although ZnPP might alter the BC contractility through its heme oxygenase–independent actions involving activation of voltage-dependent Ca current[28] and/or through its direct inhibitory action on soluble guanylate cyclase,[13] the effects of this reagent under current experimental conditions appear to be ascribable to its action on heme oxygenase: First, a paucity of voltage-dependent Ca^{2+} channel was reported previously in rat hepatocytes.[29] Second, as seen in the current study, ZnPP can increase $[Ca^{2+}]_i$ even in the absence of Ca^{2+} in an extracellular space. Finally, treatment with ZnPP evoked only little, if any, alteration in the cellular cGMP contents. The present results thus suggest physiological significance of the heme oxygenase–CO system in hepatocytes as an endogenous regulator of BC function.

Our observation that BAPTA-AM attenuated the ZnPP-induced activation of BC contraction suggests involvement of Ca^{2+} in the activation processes. Furthermore, the ZnPP application actually evoked a marked increase in intracellular Ca^{2+} concentration that was abolished by a further supplement of CO at micromolar levels. Although we were not able to demonstrate BC contraction simultaneously with the alterations in the intracellular Ca^{2+} concentrations for technical reasons, the findings suggest that suppression of endogenous CO generation could elicit the activation of BC contraction through the mechanisms involving intracellular Ca^{2+} mobilization. These results together with the fact that nitric oxide interferes with the Ca^{2+} signals through a cGMP-dependent inhibition of the inositol triphosphate production[5] led us to speculate that endogenously generated CO up-regulates cGMP, and thereby suppresses the intracellular Ca^{2+} mobilization in the couplets. This hypothesis is, however, not tenable, because the cellular cGMP levels were not altered by the administration of micromolar levels of CO, which is effective in changing BC contractility under the current experimental conditions. The current findings thus support a concept that CO can regulate intracellular Ca^{2+} mobilization, and hence BC contraction, through a cGMP-independent mechanism.

The intracellular target molecule(s) to which CO binds has not been identified yet. However, the present results showing that clotrimazole and metyrapone suppressed the ZnPP-elicited activation of BC contraction suggest that inhibition of cytochrome P450–dependent monooxygenases is a putative

TABLE 3. Changes in cGMP Contents in Cultured Hepatocytes Treated With CO, SNAP, ZnPP, and FePP

Groups	cGMP Contents (pmol/10^7 cells)
Control	0.36 ± 0.07
100 μmol/L SNAP	3.04 ± 0.60*
1 μmol/L ZnPP	0.38 ± 0.04
1 μmol/L FePP	0.45 ± 0.07
2 μmol/L CO	0.39 ± 0.07
20 μmol/L CO	0.47 ± 0.07

*$P < .05$ as compared with the control value. Data represent means ± SD of five separate experiments.

mechanism by which CO can alter the cell function: This enzyme reaction is known to involve arachidonic acid–dependent generation of biologically active eicosanoids including epoxyeicosatrienoic acids, which stimulate intracellular Ca^{2+} mobilization.[30,31] Because CO shares the interfering action on heme coordination of the enzyme with clotrimazole and metyrapone, it is not unreasonable to speculate that CO can attenuate the ZnPP-induced increase in $[Ca^{2+}]_i$ and BC contractility through the inhibition of cytochrome P450 enzymes.

The mechanism by which CO inhibits cytochrome P450 monooxygenases involves its binding to the reduced form of heme.[30] To assess the inhibitory effect of CO on the enzyme activity, we used a fluorometric assay that can be applied to isolated cultured hepatocytes in which local oxygen tension in the intracellular space is extremely low as compared with that in microsomal samples exposed to ambient conditions. The finding obtained by this method provided evidence that as small as 1 to 2 μmol/L CO can significantly inhibit the enzyme activity in cultured hepatocytes. Under the present experimental conditions, we were unable to demonstrate that elimination of CO by ZnPP increases the apparent activity of the cytochrome P450–dependent deethylation in isolated hepatocytes. Obviously, refinement of the methodology to overcome technical problems involving a large variation of the fluorescence response among different hepatocyte preparations should be necessary to fully address whether endogenously generated CO can actually exert its inhibitory action on the enzyme activity. Further investigation is obviously required to demonstrate that CO could modulate specific types of cytochrome P450s that participate in regulation of intracellular calcium mobilization (e.g., cytochrome P450–dependent eicosatrienoic acid epoxygenase), and thereby alter the cell function.[17]

Whether the increased frequency of BC contraction evoked by endogenous CO suppression may contribute to the increasing efficiency of bile transport remains to be elucidated; the blockade of CO generation reduced the effective stroke changes in the canalicular area. Actually, we have shown that elimination of endogenously generated CO by ZnPP did not increase the baseline bile output in the absence of taurocholate, but evoked a remarkable choleresis when the baseline bile output was prestimulated with taurocholate.[7] The current findings together with such previous data tempted us to hypothesize that the endogenous CO suppression merely activates unnecessary BC contractions and does not help effective bile transport until the steady-state bile formation is fully stimulated in hepatocytes. In other words, we propose that constitutive levels of CO generation are necessary to maintain physiologically optimal periodicity of BC contraction to guarantee the efficiency of the bile transport at a canalicular level.

On the other hand, excessive amounts of CO appear to function as a cholestatic factor; exogenously applied CO at 20 μmol/L abolished autonomic contraction of BC and elicited its maximum dilatation. The CO-induced BC relaxation appears to be a consequence of functional alterations, but not that of irreversible cell damages, because the cells did not display bleb formation even under exposure to 20 μmol/L CO, a concentration that did not suppress the basal oxygen consumption in cultured hepatocytes.[32] It is, however, quite unknown whether such a high concentration of CO actually occurs *in vivo*, considering that concentrations of CO detectable in the venous perfusate of the isolated perfused liver are as small as several hundred nanomolar levels.[6] Stoichiometric evaluation of CO generation and its correlation to hepatobiliary function should be necessary under various disease conditions in which hepatocytes can be exposed to excessive CO generation to establish its pathological significance as an endogenous cholestatic factor. Attempts to elucidate the whole picture of the novel biological action of CO generated by heme oxygenase are currently underway in this laboratory.

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