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EFFECT OF BILE ACIDS ON INTRACELLULAR CALCIUM IN ISOLATED RAT HEPATOCYTE COUPLETS

NANCY THIBAULT* and FRANÇOIS BALLET
Drug Safety Department, Rhône-Poulenc Rorer S.A. BP 14, 94 403 Vitry sur Seine cedex, France

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Abstract—The effects of bile acids on cytosolic free calcium ($[Ca^{2+}]_i$) were studied in single isolated rat hepatocyte couplets using a scanning laser cytometer and the fluorescent dye, indo-1. Cholestatic bile acids, chenodeoxycholate (CDC) and taurolithocholate (TLC) increased $[Ca^{2+}]_i$ in a dose-dependent manner. Choleretic bile acids, tauroursodeoxycholate (TUDC) and taurocholate (TC), did not induce any change in $[Ca^{2+}]_i$ except TC at very high doses. Treatment with TUDC added concomitantly with CDC or TLC significantly decreased the rise in $[Ca^{2+}]_i$ induced by bile acids. These results, obtained with a polarized hepatocyte model that secretes bile, confirmed that cholestatic bile acids increase $[Ca^{2+}]_i$ and showed that TUDC inhibits this phenomenon. These data are in agreement with a key role of intracellular calcium in cholestasis.

Cholestasis is one of the main manifestations of hepatotoxicity and is functionally defined as a decrease in canalicular bile flow [1–4]. Mono- and dihydroxylated bile salts such as chenodeoxycholate (CDC†) and taurolithocholate (TLC) produce cholestasis [1, 3, 5]. However, the cellular mechanism of this effect is not known. A possible role of calcium in bile acid-induced cholestasis has been suggested but there are some discrepancies regarding the mechanism of calcium modifications. It has been suggested that cholestatic bile acids induce an elevation of cytosolic calcium ($[Ca^{2+}]_i$) in isolated rat hepatocytes via a release from the endoplasmic reticulum [6, 7], while others have provided data suggesting that extracellular calcium is the primary source of the increase in cytosolic calcium [8]. Recently, another report [9] showed that release of calcium from the endoplasmic reticulum was not a relevant mechanism for bile acid-induced cholestasis in isolated perfused rat liver. In view of these differences, it was important to investigate, under other experimental conditions, the effect of CDC and TLC on $[Ca^{2+}]_i$ in comparison with the choleretic bile acids, taurocholate (TC) and tauro-ursodeoxycholate (TUDC). TUDC, the taurine conjugate of ursodeoxycholic acid which has been demonstrated to be an effective treatment for chronic cholestatic liver diseases [10], has been shown to prevent liver lesions induced by TLC in *in vivo* studies in the rat as well as in the isolated rat liver [11–13]. However, the mechanism of the protective effect of TUDC is unknown. We showed recently that TUDC inhibits actin filament alteration induced by TLC in isolated rat hepatocyte couplets [14]. It has also been shown in a study on human neutrophils that TUDC prevents the $[Ca^{2+}]_i$ increase induced by formyl-methionyl-leucyl-phenylalanine suggesting that this mechanism could explain its protective effect [15], but this has not been investigated yet in hepatocytes. These observations prompted us to examine the effect of TUDC on calcium alterations induced by cholestatic bile acids in hepatocytes.

In this study we investigate the effect of bile acids on $[Ca^{2+}]_i$ using isolated rat hepatocyte couplets (IRHC). In this model, pairs of hepatocytes enclose a functional canalicular space which allows the study of bile secretion and the vectorial transport of bile acids [16–20]. We used this model in conjunction with a scanning laser cytometer, a *new* technique which provides an accurate quantification of fluorescent probes in single adherent cells. We firstly assessed the effects of bile acids on $[Ca^{2+}]_i$. Secondly, we studied the effect of TUDC on the modifications induced by the two cholestatic bile acids.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (200–250 g) from Charles River (Saint Aubin les Elbeuf, France) were used. They had free access to food and water.

Reagents. Collagenase (type I) was obtained from Boehringer Mannheim GmbH (Meckeneim, Germany) and ketamine hydrochloride from Rhône Merieux (Lyon, France). Leibovitz 15 (L-15) and fetal calf serum were supplied by Gibco (Grand Island, NY, U.S.A.). Indo-1-AM, indo-1 free and the calcium calibration buffer (kit II) were obtained from Molecular Probes (Junction City, OR, U.S.A.). All other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and were of the highest purity commercially available.

Preparation and culture of IRHC. After anesthesia with ketamine hydrochloride (125 mg/kg of body weight), the non-recirculating collagenase perfusion of Seglen [21] modified by Graf *et al.* [18] was used. The liver was perfused via the portal vein with 500 mL of a Ca^{2+}/Mg^{2+}-free Hepes buffer solution

* Corresponding author. Tel. (33) 1 45 73 75 76; FAX (33) 1 45 73 80 94.

† Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium; IRHC, isolated rat hepatocyte couplets: CDC, chenodeoxycholate; TLC, taurolithocholate; TUDC, tauroursodeoxycholate; TC, taurocholate; ACAS, Anchored Cell Analyser and Sorter; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetracetic acid; AVP, vasopressin.

at 37°, at a flow rate of 40 mL/min and then dissociated with 250 mL of the same buffer containing 0.03% (w/v) collagenase, 5 mM $CaCl_2$ and 0.8 U of trypsin inhibitor/U of trypsin activity in the collagenase at a flow rate of 20 mL/min. The liver was then excised, minced in L-15 supplemented with 12 mM $NaHCO_3$, 10% fetal calf serum, filtered sequentially through 2 sizes of nylon mesh (60 and 30 μm) and allowed to settle by gravity in a conical centrifuge tube for three successive 10-min periods to sediment hepatocytes selectively. Viability of the cells was 92 ± 3% as measured by Trypan blue exclusion. The cells were plated at 10^6 per 35-mm dish in 2 mL of the medium and placed in a 37°, 5% CO_2 incubator for 3 hr.

Measurement of intracellular calcium. $[Ca^{2+}]_i$ was measured with a scanning laser cytometer, ACAS 570 (Anchored Cell Analyser and Sorter), equipped with a 5 W argon ion laser. The optical system of the ACAS 570 is designed to excite a sample with a laser beam at one wavelength, and to monitor emission at two wavelengths, simultaneously using two photomultiplier tubes. Consequently, fluorescent calcium-chelating indicator, such as indo-1, with a single excitation at 356 nm, and dual emission (indo-1-free at 485 nm and indo-1 bound to calcium at 405 nm) can be used to monitor changes in $[Ca^{2+}]_i$. Cells were loaded for 45 min at 37° with 2 μM indo-1-AM in L-15 supplemented with 0.02% Pluronic F-127 which reduces the problem of incomplete hydrolysis of the acetoxymethyl esters to the chelator [22]. After washing, hepatocytes were incubated for another 15-min period at 37° to allow complete hydrolysis of indo-1-AM to indo-1. Couplets were chosen at random and scanned for fluorescence. After the basal concentration was obtained (two or three scans), vasopressin (AVP) and bile acids were added and the scanning was continued for 10 min (TLC was dissolved in a final medium containing 1% dimethyl sulfoxide and a control culture was performed with the same dimethyl sulfoxide concentration). The ratio F_{405}/F_{485} was calculated for each cell and the calcium concentration was derived from a standard curve which was generated using free acid Indo-1 and varying concentrations of standard calcium.

Statistical methods. Data are expressed as means ± SD. For each separate isolation, differences in mean values were compared using the paired *t*-test. N indicates the number of couplets studied.

RESULTS

Morphology

Three hours after plating, 25 ± 8% of the cells had formed IRHC and showed a closed canalicular space (bile canaliculus structure) of variable size (2–6 μm in diameter). After 8 hr in culture, the cells flattened down and the canalicular spaces collapsed. Therefore, studies were performed between 3 and 8 hr of culture as described previously [17].

Effect of vasopressin and bile acids on cytosolic $[Ca^{2+}]_i$

Basal $[Ca^{2+}]_i$ from hepatocyte couplets averaged 170 ± 42 nM (N = 30) from five separate isolations) between 3 and 6 hr after plating. Couplets were first treated with four concentrations of AVP (0.1, 1, 10 and 100 nM). Except for 0.1 nM, all these concentrations provoked an immediate, concentration-dependent and prolonged increase in $[Ca^{2+}]_i$ (Fig. 1A). Under our experimental conditions, we observed that this concentration-dependent effect was due to the number of couplets responding to AVP which increased with the concentration used. CDC and TLC increased $[Ca^{2+}]_i$ in a dose-dependent manner within 20 sec of addition of bile acids, with a peak value reached within 60 sec. While this effect was slowly reversible within 4 or 5 min with a 125 μM concentration for CDC (Fig. 1B) and 250 μM for TLC (Fig. 1C), there was a secondary and irreversible rise at higher concentrations. In contrast, neither TC nor TUDC were found to induce any changes in $[Ca^{2+}]_i$. Nevertheless, TC at a very high concentration (>3 mM) induced a transient increase in $[Ca^{2+}]_i$ (Fig. 1D).

Effect of bile acids on $[Ca^{2+}]_i$ *in the absence of extracellular* Ca^{2+}

The effects of bile acids in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$) were studied to determine the role of calcium influx in this response. The external calcium concentration was reduced from 1.24 mM to less than 6 μM by adding 1.24 mM EGTA to the medium 15 sec prior to the studied agents in order to abolish Ca^{2+} influx into the hepatocytes, without altering internal Ca^{2+} stores [6]. Basal $[Ca^{2+}]_i$, in the absence of $[Ca^{2+}]_o$, decreased (120 ± 39 vs 170 ± 42 nM) after 2 min of exposure, probably because these experimental conditions deplete internal stores. Under these conditions, addition of CDC or TLC produced the same initial rise in $[Ca^{2+}]_i$ that was observed with extracellular calcium, but the return to the basal level was more rapid (within 2 min) (Fig. 2A and B).

Effect of TUDC on bile acid-induced $[Ca^{2+}]_i$

When TUDC was added concomitantly at an equimolar concentration (125 μM) with CDC or TLC to the culture medium, the initial rise in $[Ca^{2+}]_i$ during the first 30 sec was still observed. However, a 40–60% $[Ca^{2+}]_i$ decrease was observed from 60 sec as compared to with TLC and CDC alone. At higher concentrations of CDC and TLC, TUDC was ineffective on the bile acid-induced increase in cytosolic $[Ca^{2+}]_i$.

DISCUSSION

In the present study, we used IRHC to investigate the effect of bile acids on intracellular calcium homeostasis. Isolated hepatocytes do not secrete bile in a closed canalicular space and therefore do not mimic the intracellular and canalicular changes that occur when bile flow is inhibited. In contrast, IRHC reform canalicular structures and are particularly adapted to bile acid studies since bile formation requires polarized liver cells to transport bile acids from sinusoidal blood, across the basolateral membranes, to the apical membranes

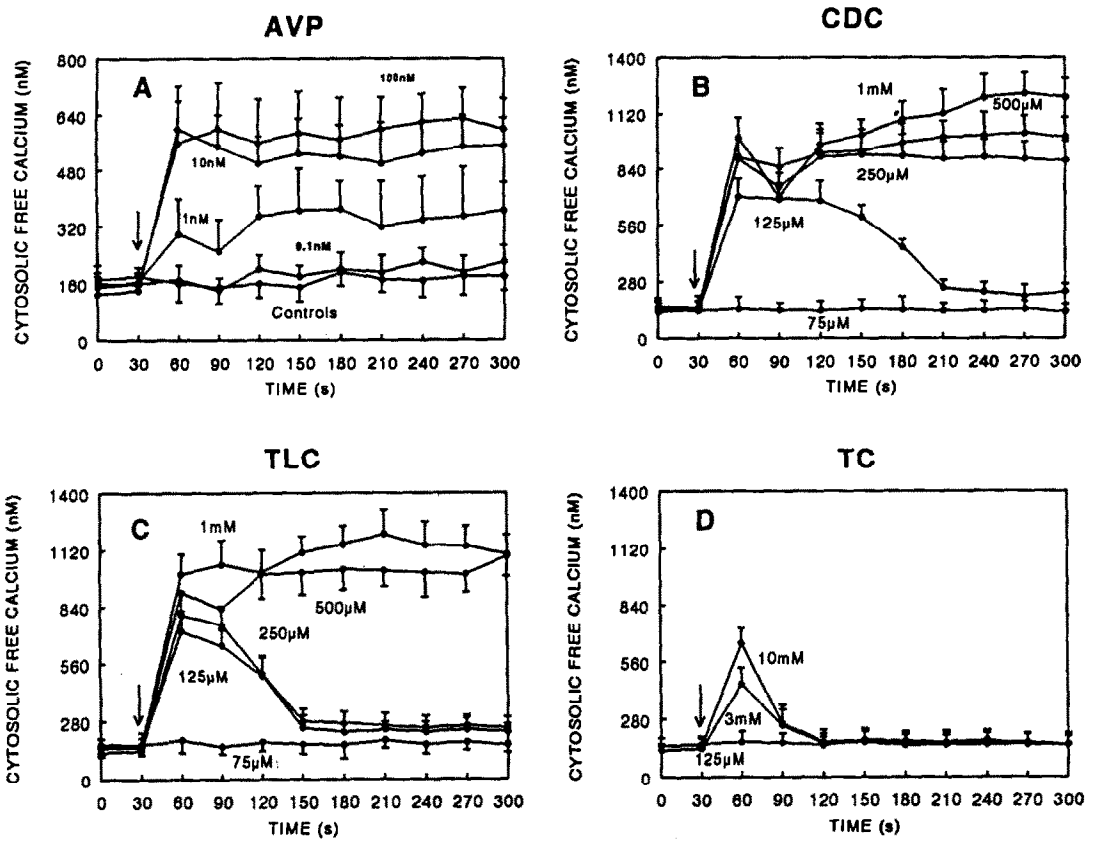

Fig. 1. Effect of increasing concentrations of AVP (A), CDC (B), TLC (C) and TC (D) on cytosolic $[Ca^{2+}]_i$ in IRHC from five different isolations (N = 30). Results are given as the means ± SD of five experiments. Arrows indicate when the compounds were added to the medium.

where they are excreted into the lumen of the bile canaliculus [4].

The use of a scanning laser cytometer with the newly available fluorescent Ca^{2+} chelator, indo-1, allowed us to perform calcium measurements in single hepatocyte couplets. Fura-2, indo-1 and fluo-3 contain a calcium-sensitive binding site modelled on the well-known chelator, EGTA [23–25]. This steric arrangement represents significant improvement over quin-2 since it gives a better selectivity for Ca^{2+} over the most serious competing ion, Mg^{2+}. Furthermore, their increased brightness fluorescence, due to their greater absorption coefficient and quantum yield [25], can be used to decrease intracellular dye loading. The absorption shift of fura-2 to shorter wavelengths on Ca^{2+} binding can be measured in cells by changes in the excitation spectrum between 300 and 400 nm while monitoring the emission at about 510 nm. The fluorescence emission of indo-1 shifts from about 490 nm in Ca^{2+}-free medium to about 405 nm when saturated with Ca^{2+} [25]. These features allow $[Ca^{2+}]_i$ to be deduced from the ratio of amplitudes at a pair of excitation wavelengths (fura-2) or emission wavelengths (indo-1) and then elimination of most of the variations due to the instrumental fluctuations or to photobleaching and changes in dye content of the cell. These are significant advantages for fura-2 and indo-1 over their prototype, quin-2, or other fluorescent probes like fluo-3 which responds to calcium with only an increase in fluorescence intensity, without a shift in excitation or emission. Although fura-2 and indo-1 have interchangeable uses, in this study, we chose indo-1 for several reasons: (i) its single excitation wavelength is suitable for the argon ion laser of the ACAS, and the two emissions are easy to monitor with its dual detector system. (ii) Furthermore, indo-1, in comparison with fura-2, permits a decrease by 50% in the amount of UV light to which the cell must be exposed for excitation of the dye while maintaining the benefits of the fluorescence ratio imaging. Therefore, indo-1 offers many advantages over fura-2 for scanning laser cytometry.

Basal $[Ca^{2+}]_i$ levels in our study are similar to those reported in the literature in isolated rat hepatocytes [6–8]. The calcium response induced by AVP is similar to that reported under different experimental conditions [26–28] and further confirms the validity of the model and the technique used to study the intracellular kinetics.

We demonstrated that addition of bile acids to IRHC at low doses causes a rapid and transient increase in $[Ca^{2+}]_i$. Removal of extracellular calcium had no effect on the initial increase in $[Ca^{2+}]_i$ induced

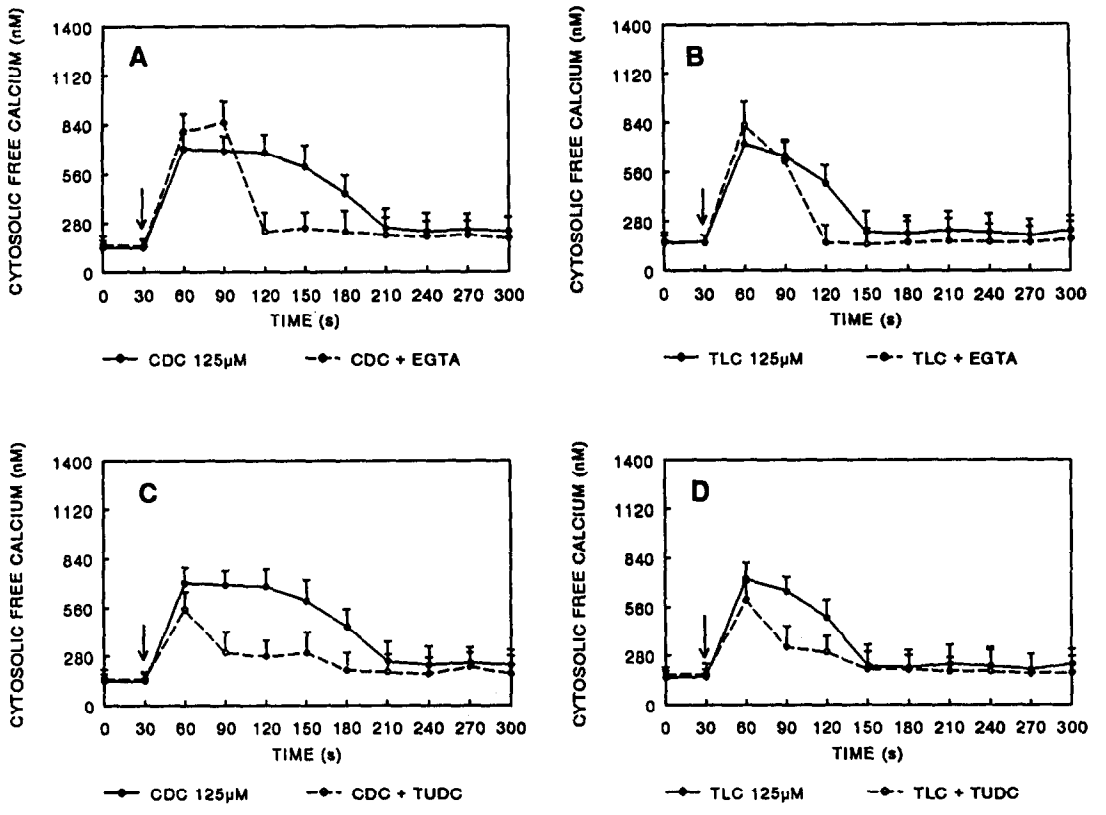

Fig. 2. Effect of EGTA and TUDC on the calcium increase induced by 125 μM CDC (A, C) and TLC (B, D). The calcium chelator EGTA (1.24 mM) added 15 sec before the agents did not prevent the $[Ca^{2+}]_i$ rise, since TUDC added concomitantly with CDC and TLC inhibits it. Results are given as the means ± SD of five experiments (N = 30). Arrows indicate when the compounds were added to the medium.

by bile acids, which suggests that initially an intracellular source of calcium is involved as hypothesized by Combettes *et al.* [6, 7].

The return to basal levels of calcium was accelerated, probably because Ca^{2+} removal from the medium suppresses the resting calcium influx. This phenomenon also suggests that influx of calcium from the extracellular medium could be responsible for the late phase of the bile acid-induced $[Ca^{2+}]_i$ increase.

At higher concentrations (>250 μM), mono- and dihydroxy bile acids induced a secondary and irreversible rise in $[Ca^{2+}]_i$. This prolonged elevation in $[Ca^{2+}]_i$ could be explained by the fact that cytotoxicity occurs at these concentrations. Under these conditions, hepatocytes probably lose their ability to pump calcium out of the cell or back into storage compartments and this disturbance probably initiates a sequence of events leading to cell death [29]. Indeed, cell death was easily observable by phase contrast microscopy (cell surface blebbing, loss of contrast, detachment of the support). TC, a choleretic bile salt which becomes cholestatic at high doses [5], increases $[Ca^{2+}]_i$ in IRHC at very high concentrations without any significant cytotoxicity. This further reinforces the hypothesis that the rise in $[Ca^{2+}]_i$ is involved in bile acid-induced cholestasis. Co-treatment with TUDC, a hydrophilic bile acid which has been shown to prevent bile acid-induced cholestasis [11–13], did not modify the initial rise in $[Ca^{2+}]_i$ but significantly decreased $[Ca^{2+}]_i$ after 60 sec.

This result is in accord with a study showing that TUDC inhibits the $[Ca^{2+}]_i$ increase in human neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine [15], suggesting a role for $[Ca^{2+}]_i$ in the TUDC protective effect. Nevertheless, at the present stage of this study, the mechanisms of the effect of TUDC on intracellular calcium under our experimental conditions are unclear. Three hypotheses can be considered: (i) a specific effect of TUDC on calcium homeostasis: either an inhibition of extracellular calcium influx or a stimulation of canalicular calcium secretion as demonstrated in the dog *in vivo* as in the isolated perfused rat liver [30, 31]. (ii) Competition with cholestatic bile acids either at the level of the carrier system [32–34], or of the intracellular binding sites [6, 7]. (iii) Increased excretion of cholestatic bile acids into the canaliculus due to the choleretic properties of TUDC [13].

We have shown previously that TLC and CDC increase pericanalicular actin in IRHC and that this

phenomenon is inhibited by TUDC [14]. Therefore, it is tempting to speculate that this effect could be related to $[Ca^{2+}]_i$ changes. Other studies are needed to clarify the relationship between intracellular calcium, pericanalicular actin microfilaments and cholestasis.

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