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Effect of tributyltin chloride on the release of calcium ion from intracellular calcium stores in rat hepatocytes

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Abstract

The effects of tri-*n*-butyltin chloride (TBT), an environmental pollutant, on the release of Ca^{2+} from intracellular stores were investigated in isolated rat hepatocytes. Isolated hepatocytes permeabilized with digitonin were suspended in solution, and the concentration of extracellular Ca^{2+} was measured, using a fluorescent Ca^{2+} dye, fura-2. In the solution containing permeabilized hepatocytes that had been preincubated with 4.0 μM TBT for 30 min, the extracellular Ca^{2+} concentration was high, but the inositol 1,4,5-trisphosphate ($InsP_3$)-induced increase in Ca^{2+} concentration was suppressed, suggesting that the extracellular release of Ca^{2+} in response to TBT treatment was from intracellular stores. Images of the Ca^{2+} concentration in the intracellular stores of primary cultured hepatocytes loaded with fura-2 were obtained after digitonin-permeabilization, using digitalized fluorescence microscopy. The permeabilized hepatocytes that had been preincubated with 4.0 μM TBT for 30 min had a very low fura-2 fluorescence ratio (340/380 nm), suggesting that stored Ca^{2+} was released. When the hepatocytes were treated with 4.0 μM TBT after digitonin-permeabilization, the decrease in the fura-2 fluorescence ratio was very small. However, when the permeabilized hepatocytes were incubated with 4.0 μM TBT and 2.0 μM NADPH, the decrease was enhanced, raising the possibility that TBT might be metabolized to the active form(s), thus releasing Ca^{2+} from intracellular stores. When the hepatocytes were preincubated with 0.1 μM TBT for 30 min and then were permeabilized, the fura-2 fluorescence ratio was almost the same as that in the control permeabilized hepatocytes. However, the $InsP_3$-induced decrease in the fluorescence ratio was suppressed significantly in the permeabilized hepatocytes. These results suggest that TBT released Ca^{2+} from the intracellular stores at high concentrations, and suppressed the $InsP_3$-induced Ca^{2+} release at non-toxic low concentrations. It is probable that the latter effect was responsible for the previously reported suppression of Ca^{2+} response induced by hormonal stimulations (Kawanish *et al*., Toxicol Appl Pharmacol 1999;155:54–61).

Keywords: Hepatocyte; Tributyltin; Calcium; Inositol 1,4,5-trisphosphate; Fluorescence microscopy

1. Introduction

Tributyltin compounds have been used as molluscicides; as antifoulants on boats, ships, quays, buoys, crab pots, fish nets, and cages; as wood preservatives; as slimicides on masonry; as disinfectants; and as biocides for cooling systems, power station cooling towers, pulp and paper mills, breweries, and leather processing and textile mills. Such widespread use of this compound has caused increasing amounts to be released into the environment. Tri-*n*-butyltin compounds have also been shown to be ubiquitous in the aquatic environment [1,2]. Thus, the toxicity of these compounds has been investigated widely, and *in vivo* toxicity has been well established [2,3]. The most characteristic toxic effect of tri-*n*-butyltin is on the immune system: due to its effects on the thymus, tri-*n*-butyltin impairs cell-mediated functions [4,5]. Tri-*n*-butyltin is also a potent skin irritant and a powerful eye irritant [1]. In addition, tri-*n*-butyltin induces hepatocellular necrosis and inflammatory changes in bile ducts [6–9]. It also has structural effects on endocrine organs, particularly the pituitary, adrenal glands, and thyroid, which, in turn, change the circulating concentrations of hormones such as thyroxine, thyroid-stimulating

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Abbreviations: TBT, tri-*n*-butyltin chloride; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $InsP_3$, inositol-1,4,5-trisphosphate; DBT, di-*n*-butyltin chloride; and KRH, Krebs Ringer HEPES buffer.

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hormones, luteinizing hormone, and insulin. Tri-*n*-butyltin is also suspected of being an endocrine-disrupting chemical [10].

Despite these numerous investigations, the underlying mechanisms of the toxicity of tri-*n*-butyltin in organisms remain unknown. Tri-*n*-butyltin is known to inhibit oxidative phosphorylation and to suppress energy production, effects that may be related to its cytotoxicity [11–13]. It has also been reported that the compound reduces cyclic AMP production [14] and changes Ca^{2+} metabolism [15,16] in isolated rat thymocytes. The immunotoxic action of tri-*n*-butyltin could be caused, in part, by cytoskeleton modifications in addition to perturbation of thymocyte Ca^{2+} homeostasis, both of which may be linked to the apoptosis of thymus cells [17]. Corsini *et al*. [18] have reported that in keratinocytes tributyltin induces Ca^{2+} mobilization, which is the triggering event for the production of reactive oxygen species. Tri-*n*-butyltin has been shown to induce calcium overload in rat pheochromocytoma cells [19] and rainbow trout hepatocytes [20,21]. In a previous study, we also demonstrated that 4.0 μM TBT increased intracellular Ca^{2+} concentration and then induced cell death in primary cultured hepatocytes [22]. In the same study, we found that 0.1 μM TBT, a concentration that does not produce toxic symptoms in hepatocytes, suppressed the Ca^{2+} response induced by hormonal stimulations. Because Ca^{2+} is involved in signal transduction pathways regulating various cellular activities, perturbation of Ca^{2+} homeostasis at the cellular level may cause a variety of effects depending on the critical concentrations of organotin compounds at the target organs.

In this report, using the fluorescent Ca^{2+} indicator, fura-2, and digitalized fluorescence microscopy, we examined the effect of TBT on the release of Ca^{2+} from intracellular stores and the Ca^{2+} concentration in those stores. The results clearly show that at high concentrations of TBT Ca^{2+} is released from intracellular stores, whereas at low concentrations, there is suppression of Ca^{2+} release by TBT. Suppression was even observed at 0.1 μM TBT, a concentration that is found in the livers of marine mammals from several locations [2,23–25].

2. Materials and methods

2.1. Chemicals

Collagenase and bovine serum albumin were obtained from Boehringer Mannheim. Collagen was purchased from the Koken Co., Ltd. Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes Inc., and $InsP_3$ was obtained from Wako Chemical Reagent. Digitonin, HEPES, Tris, and Chelex 100 were from the Sigma Chemical Co. TBT and DBT were purchased from the Tokyo Kasei Kogyo Co., Ltd. TBT and DBT were dissolved in ethanol at appropriate concentrations. Then the ethanol solutions were added to the incubation solutions so that the final ethanol concentration was 0.4%, a concentration that had no effect on the results. All other reagents were commercial products of the highest grade of purity available.

2.2. Hepatocyte isolation

Hepatocytes were isolated from male Sprague-Dawley rats (4- to 6-weeks-old), obtained from the Nippon SLC Co., by collagenase digestion as described previously [26]. Cell viability, judged by trypan blue exclusion, was more than 90%. When the hepatocytes were cultured, isolated cells were diluted to 5.0×10^5 cells/mL in Williams' medium E supplemented with 5% fetal bovine serum, 10 nM dexamethasone, and 100 nM insulin. Aliquots of 1 mL were cultured on collagen-coated 12.5-mm-radius glass coverslips inside 35 × 10-mm plastic Petri dishes. Hepatocytes were cultured overnight in humidified 5% CO_2 and 95% air at 37°.

2.3. Measurement of the release of Ca^{2+} from digitonin-permeabilized hepatocytes

Isolated hepatocytes (2×10^6 cells) were washed four times with nominally Ca^{2+}-free solution (120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 20 mM HEPES/Tris, pH 7.4), suspended in 2 mL of buffer A (a solution containing 120 mM KCl, 1 mM KH_2PO_4, 10 mM NaCl, 20 mM HEPES/Tris, pH 7.2, mixed with Chelex 100 to remove contaminating Ca^{2+}), and incubated for more than 10 min at 37°. The supernatant obtained was supplemented with antipain (1 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), Ruthenium Red (1 μM), MgATP (2 mM), phosphocreatinin (5 mM), and creatine kinase (5 U/mL). The cell suspension was placed in a quartz cuvette in a JASCO CAF-100 fluorometer (Jasco), and digitonin (25 mg/mL) was added with stirring. After about 5 min, fura-2 (1.5 μM) was added to the cell suspension. Fluorescence intensities were measured at alternating excitation wavelengths of 340 and 380 nm through a BA500 IF filter, and the fluorescence ratio was calculated. After the procedure, Ca^{2+} was added to a concentration of 1 mM, and the fluorescence intensity was measured under a Ca^{2+}-saturated condition (R_{max}). EGTA/Tris solution (pH 8.5) was then added to a concentration of 10 mM, and fluorescence intensities were measured under a Ca^{2+}-free condition (R_{min}). The Ca^{2+} concentration was calculated from the R_{min} values, the R_{max} values, and the K_d value (224 nM) of fura-2 following an equation in Grynkiewicz *et al*. [27].

2.4. Measurement of Ca^{2+} in the intracellular Ca^{2+} stores using compartmentalized fura-2

Hepatocytes were cultured overnight on collagen-coated cover-glasses. The hepatocytes were incubated with 10 μM fura-2/AM, which was emulsified with pluronic F127 just before loading, in KRH solution (115 mM NaCl, 5 mM

KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 25 mM HEPES, pH 7.4) for about 2 hr at room temperature with shaking. Each coverslip was washed twice with Ca^{2+}-free solution and attached to the bottom of a 35 × 10-mm plastic Petri dish with a hole (18-mm diameter), which was then mounted in an experimental chamber fitted to the stage of a Nikon Diaphoto TMD inverted fluorescence microscope (Nikon); then buffer B [120 mM KCl, 1 mM KH_2PO_4, 10 mM NaCl, 20 mM HEPES/Tris, pH 7.2, antipain (1 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), Ruthenium Red (1 μM), MgATP (2 mM), $CaCl_2$ (250 nM), and EGTA (500 nM)] was added to the dish. Fura-2 fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm through a 410 nm dichroic reflector and a BA510 IF filter, collected by a Hamamatsu Photonics C2400-87 intensified CCD camera, and processed by a Hamamatsu Photonics ARGUS-50 image analyzer. The images collected for each wavelength of excitation (32 frames each) were averaged and corrected for background fluorescence and shading. The 340/380 nm ratio was calculated on a pixel-by-pixel basis. The mean fluorescence ratio for each cell was obtained by averaging the fluorescence ratio of each pixel within a spot of more than 6.25 μm diameter centered over the cell. The fluorescence of fura-2 is known to depend on ionic strength, pH, concentration of ions, and fluidity. We therefore did not calibrate the luminar Ca^{2+} concentration from the fluorescence ratios, since we did not know these parameters in the Ca^{2+} stores. The temperature was kept at 32°. The ratio images were obtained every 10–30 sec, unless otherwise noted.

3. Results

3.1. Detection of Ca^{2+} release using permeabilized hepatocyte suspensions

We previously reported that TBT in concentrations of more than 1.0 μM increases the intracellular calcium ion concentration in primary cultured hepatocytes and finally induces cell death. However, preincubation with 0.1 μM TBT for 30 min suppressed the calcium responses induced by hormonal stimulations, although no toxic effects were shown in the pretreated cells [22]. The hormonal stimulations enhance the production of $InsP_3$, which releases Ca^{2+} from the intracellular stores. In this previous study, we also found that $InsP_3$-production is not decreased in the TBT-pretreated cells and that the increase in $InsP_3$-production induced by hormonal stimulations is not changed. In the present study, therefore, we examined the effect of TBT on the release of Ca^{2+} from the intracellular stores.

First, we tried to detect the release of Ca^{2+} by measuring Ca^{2+} concentration in buffer A containing fura-2 and a suspension of digitonin-permeabilized hepatocytes. When $InsP_3$ was added to the suspension, Ca^{2+} concentration was increased from 105 ± 18 nM (N = 4) to about 403 ± 29 nM (N = 4) (Fig. 1A). This increase was suppressed by the presence of heparin (50 μg/mL), an inhibitor of the $InsP_3$ receptor (Fig. 1B), suggesting that the increases resulted from the release of Ca^{2+} from intracellular stores through the $InsP_3$-receptor channel. When the hepatocytes were incubated with 4.0 μM TBT for 30 min, followed by treatment with 10 μg/mL of digitonin, the Ca^{2+} concentration in the suspension solution was 305 ± 42 nM (N = 4) before the addition of $InsP_3$ and was increased to only 342 ± 40 nM (N = 4) by $InsP_3$ (Fig. 1C), suggesting that TBT also caused the release of Ca^{2+} from intracellular stores. Next, the effect of a low concentration of TBT on Ca^{2+}-release by $InsP_3$ was examined in this model. After pretreatment with 0.1 μM TBT for 30 min, the extracellular Ca^{2+} concentration in the cell suspension [48 ± 12 nM (N = 3)] was almost the same as that in the control [53 ± 12 nM (N = 3)]. The maximum Ca^{2+} concentration after the addition of $InsP_3$ was lower in the TBT-pretreated cell suspension [193 ± 18 nM (N = 3)] than in the control [210 ± 20 nM (N = 3)] (Fig. 2, A and B), but the difference was not statistically significant.

3.2. Detection of Ca^{2+}-release by imaging Ca^{2+} concentration in the intracellular stores of compartmentalized fura-2

Second, we obtained fluorescent images reflecting Ca^{2+} concentration in intracellular organelles using fura-2, and investigated the effects of TBT on Ca^{2+} stores. Primary cultured hepatocytes were incubated with fura-2/AM for about 2 hr at room temperature. The cells were then treated with 20 μg/mL of digitonin. After 5–10 min, cytoplasmic fluorescence dissipated, while fluorescence associated with intracellular organelles persisted. The fluorescence was quickly quenched by the addition of 0.2 mM Mn^{2+} and 5 μM ionomycin, but not by the addition of 0.2 mM Mn^{2+} alone, indicating that the fluorescence occurred in the organelles. When $InsP_3$ (5.0 μM) was added to the digitonin-permeabilized hepatocytes, the fura-2 fluorescence ratio (340/380 nm) was decreased in almost all of the cells (Fig. 3A). The decrease of the fluorescence ratio by $InsP_3$ was suppressed in the presence of 25 μg/mL of heparin (Fig. 3B). The addition of 0.5 μM thapsigargin also decreased the fluorescence ratio but gradually (Fig. 3C). After the addition of $InsP_3$, 2.5 μM ionomycin decreased the fluorescence ratio further (Fig. 3D).

The above results demonstrated that the decrease in the fura-2 fluorescence ratio by $InsP_3$ was due to the release of Ca^{2+} from the intracellular stores, and that Ca^{2+} was probably released through the $InsP_3$-receptor channel. Therefore, we next examined the effects of TBT-treatment on the intracellular Ca^{2+} stores. When primary cultured hepatocytes were incubated with 4.0 μM TBT for 30 min, the fluorescence ratio was already very low after the permeabilization by digitonin-treatment (Fig. 4A). The decrease was

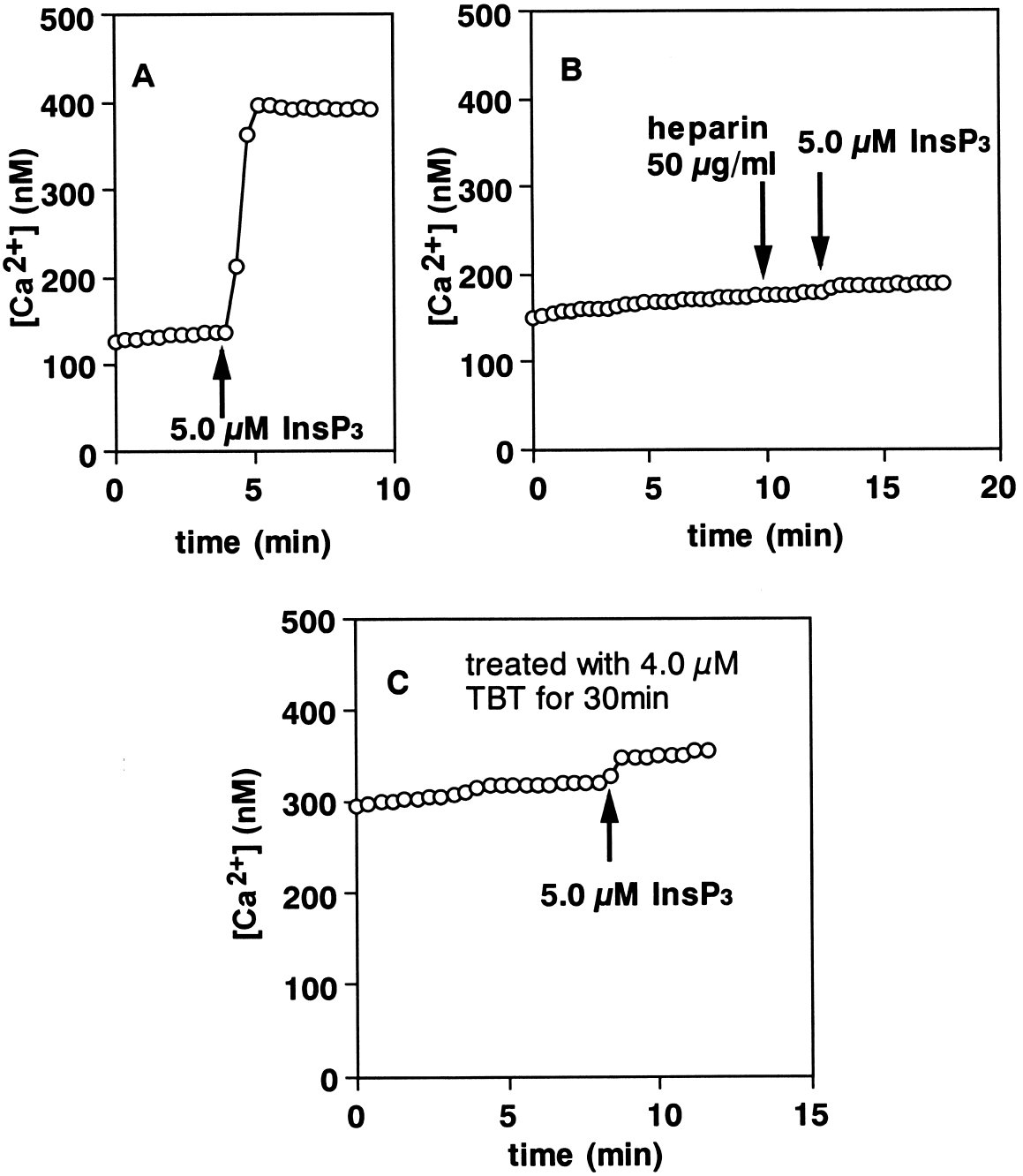

Fig. 1. Ca^{2+} concentration, monitored by fura-2, in a suspension of isolated hepatocytes permeabilized with digitonin. Shown is a typical result from 4–6 experiments. (A) Increase in Ca^{2+} concentration induced by $InsP_3$. (B) Suppression of the $InsP_3$-induced increase in Ca^{2+} concentration by heparin. (C) Change in Ca^{2+} concentration in a suspension of isolated hepatocytes treated with 4.0 μM TBT for 30 min. This concentration of TBT was toxic in the cells; however, the viability of the cells decreased by less than 10% for 30 min. Therefore, the results were not normalized.

not detected when the concentration of TBT was 0.2 μM. However, TBT at 1.0 μM seemed to decrease the fura-2 fluorescence ratio, although the effect was not significant (Fig. 4B). The results suggested that TBT releases and depletes Ca^{2+} from the intracellular stores during the 30-min incubation (Fig. 4A). On the other hand, when the hepatocytes were incubated with 4.0 μM TBT for 30 min after permeabilization by digitonin, the effect was not strong in more than three-fourths of the hepatocytes, although the fluorescence ratio decreased in the other cells (Fig. 5).

The results shown in Fig. 5 raised the possibility that the TBT-induced depletion of Ca^{2+} was not the result of a direct effect of TBT on the Ca^{2+}-stores. Nor was the depletion suppressed in the presence of heparin, an $InsP_3$ receptor channel blocker (data not shown), suggesting that TBT did not open the $InsP_3$ channel as an agonist. Because

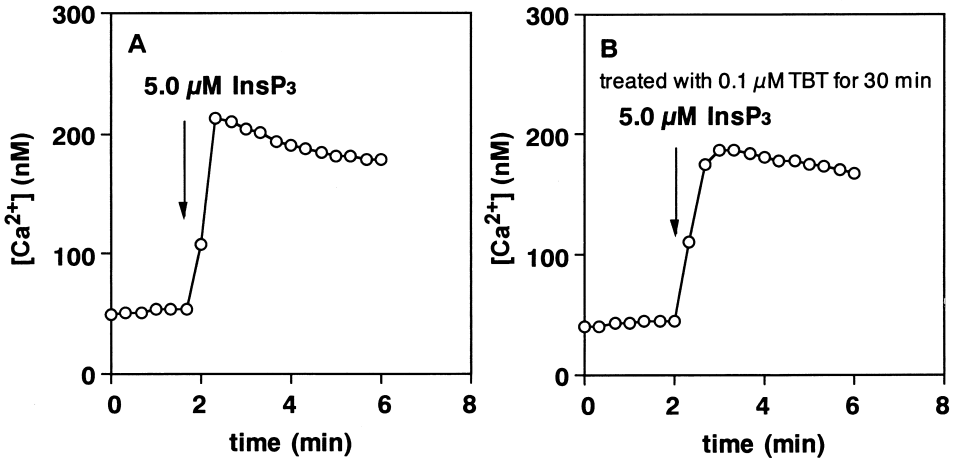

Fig. 2. Comparison of the $InsP_3$-induced increase in Ca^{2+} concentration between a suspension of (A) control isolated hepatocytes, and (B) hepatocytes pretreated with 0.1 μM TBT for 30 min. A typical result from three experiments is shown.

we previously showed that 4.0 μM TBT decreases intracellular ATP, on which Ca^{2+}-pump activity in the Ca^{2+} stores depends [22], there was a possibility that the decrease in the ATP content resulted in the Ca^{2+}-depletion. There was also a possibility that TBT was metabolized to an active substance(s) that depleted Ca^{2+} from the stores, because TBT is known to be metabolized to di-*n*-butyltin and mono-*n*-butyltin [28]. To examine the former possibility, we permeabilized hepatocytes with digitonin, then incubated them for 30 min in buffer B without ATP, and compared the fluorescence ratio to that in the hepatocytes incubated in normal buffer B. The fluorescence ratio [1.21 ± 0.08 (N = 8)] was significantly ($P < 0.01$ by Student's *t*-test) lower than that in the control cells [1.53 ± 0.10 (N = 7)], but much higher

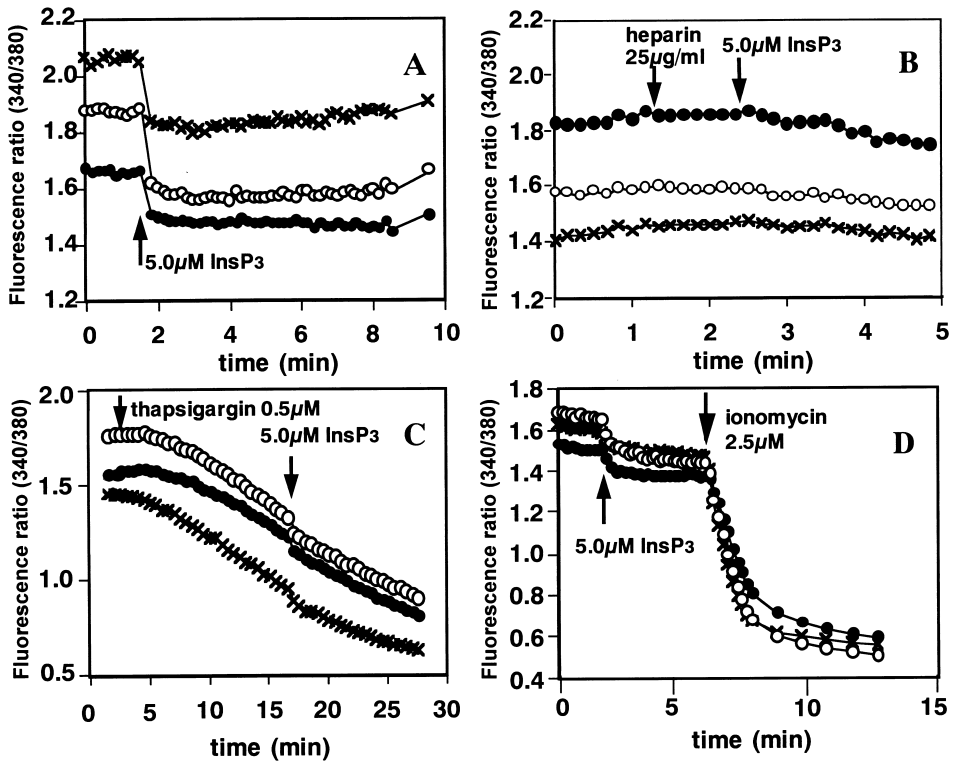

Fig. 3. Fluorescence ratio of fura-2 compartmentalized in primary cultured hepatocytes permeabilized with digitonin. Typical results from three experiments (❍, ×, ●) are shown for 18–57 cells on 3–8 different glass coverslips. (A) Decrease in the fluorescence ratio induced by $InsP_3$. (B) Suppression of the $InsP_3$-induced decrease in fluorescence ratio by heparin. (C) Decrease in the fluorescence ratio induced by thapsigargin. (D) Decrease in the fluorescence ratio induced by $InsP_3$, followed by a decrease induced by ionomycin.

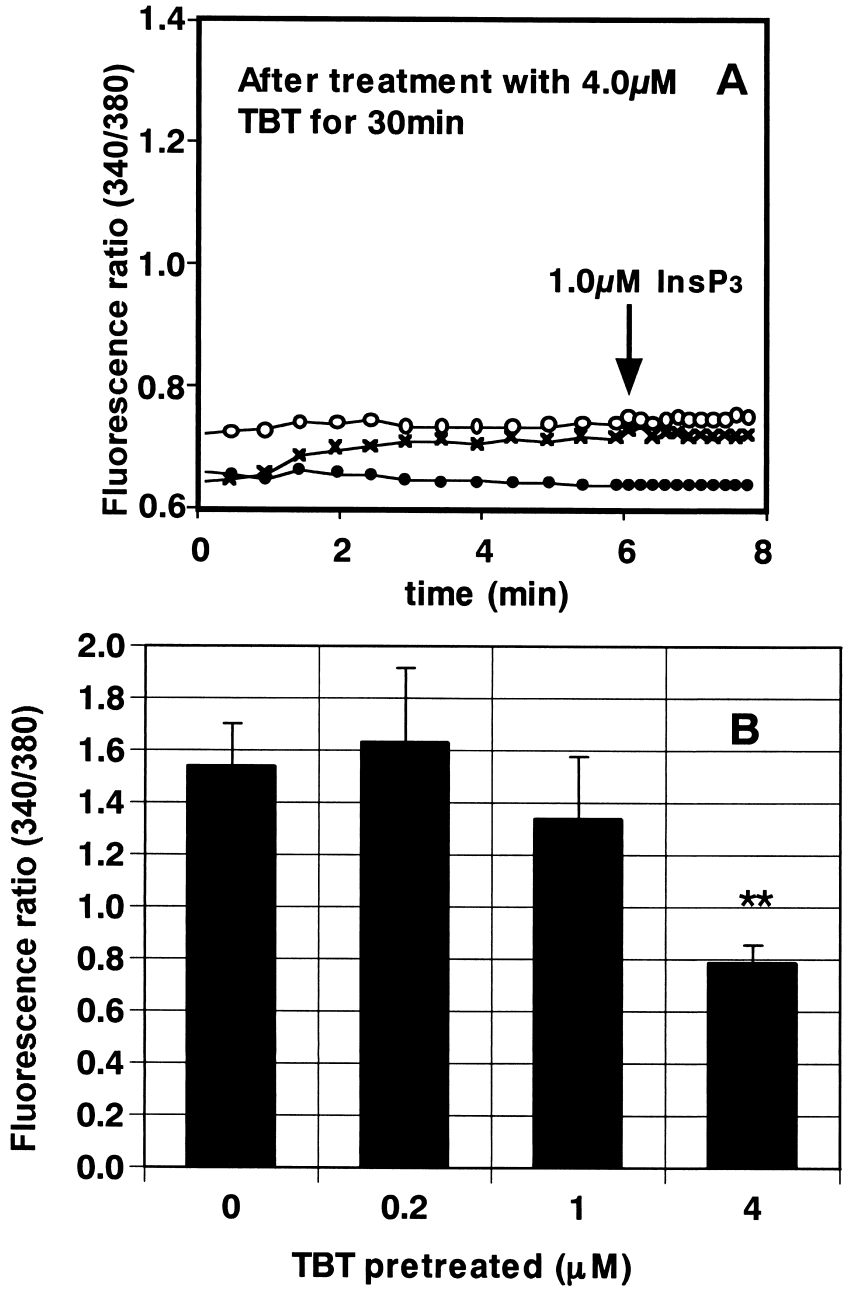

Fig. 4. Fluorescence ratio of fura-2 in permeabilized cultured hepatocytes pretreated with TBT. The hepatocytes were incubated with TBT for 30 min at 32°, permeabilized with digitonin, and then the fluorescence images were obtained. (A) Typical results from three experiments (❍, ×, ●) showing the time-dependent changes of the fura-2 fluorescence ratio after permeabilization with digitonin for 15 cells incubated with 4.0 μM TBT on 4 different coverslips. (B) Concentration-response relationship on the effect of TBT-treatment on the fura-2 fluorescence ratio in permeabilized cells. The fluorescence ratios were obtained just after permeabilization with digitonin. Results are the means ± SD of 3–15 cells. Statistical analysis was performed by Scheffe's test. Key: (**) $P < 0.01$ vs control (0 μM).

than that in the TBT-treated cells [0.68 ± 0.10 (N = 8)]. Next, we examined the latter possibility. Tri-*n*-butyltin was thought to be metabolized mainly to di-*n*-butyltin by mixed-function oxidase systems in the endoplasmic reticulum [28, 29]. Almost all of the oxidative reactions catalyzed by these enzyme systems are known to require a cofactor, NADPH. In the digitonin-permeabilized hepatocytes, intracellular NADPH should diffuse out of the cells. Consequently, it was possible that the metabolic activation of tri-*n*-butyltin was suppressed in the digitonin-permeabilized cells because of the leak of NADPH. Therefore, we examined the effect of DBT and also added NADPH to the solution in which the permeabilized cells were being incubated with TBT (Fig. 6). As shown in Fig. 6, DBT had no significant effect on the Ca^{2+}-stores, even if the hepatocytes were incubated with DBT before or after the permeabilization with digitonin. Nor did NADPH change the effect of DBT. On the other hand, when NADPH was added to the reaction mixture

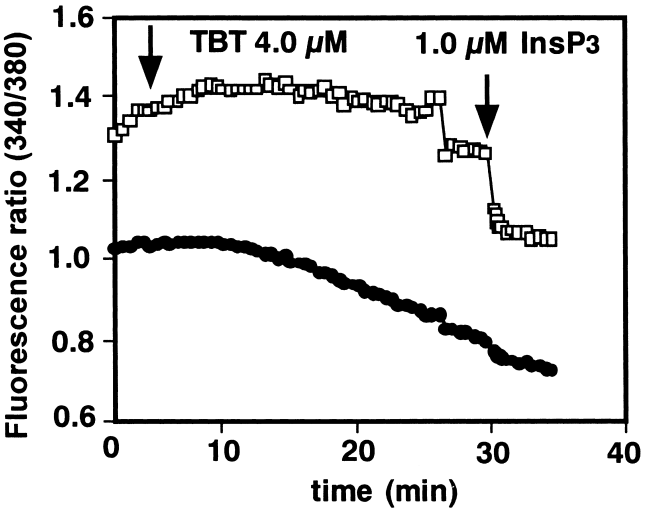

Fig. 5. Decrease by TBT-treatment of the fura-2 fluorescence ratio in permeabilized cultured hepatocytes. The hepatocytes were treated with digitonin. The changes in the fluorescence ratio at around 26 min was an artifact and caused by re-focusing. Shown are typical results of two experiments from 45 cells on 9 different coverslips. The decrease of the fura-2 fluorescence ratio was not apparent in more than three-fourths of the cells (□), although the ratio decreased in the other cells (●).

during incubation of digitonin-permeabilized hepatocytes with TBT, the fluorescence ratio was decreased, although this decrease was no greater than that in the cells treated with TBT before permeabilization.

3.3. Effects of TBT-treatment on the release of Ca^{2+} induced by $InsP_3$

In our previous paper, we reported that TBT-preincubation suppresses the hormone-induced Ca^{2+} response at low concentrations, such as 0.1 μM [22]. Therefore, we investigated the induction of Ca^{2+} release by $InsP_3$ in hepatocytes pretreated with 0.1 μM TBT for 30 min. The low concentration of TBT did not change the fluorescence ratio in the hepatocytes. We then compared the $InsP_3$-induced decrease in fluorescence ratio between the TBT-pretreated and control cells. The $InsP_3$-induced decrease in fluorescence ratio was dependent upon the concentration of $InsP_3$. The decrease was suppressed significantly in TBT-pretreated cells when 0.25 to 1.0 μM $InsP_3$ was added. The suppression was clearer when the $InsP_3$ concentration was low. Addition of 2.5 μM $InsP_3$ resulted in no observable suppression. The EC_{50} values were 0.27 and 0.42 μM in the control cells and the TBT-pretreated cells, respectively. These results clearly show that the low concentration (0.1 μM) of TBT suppressed the $InsP_3$-induced Ca^{2+} release from the intracellular stores (Fig. 7).

We also examined the concentration response relationship in the suppression by TBT-pretreatment of the Ca^{2+}-release induced by 0.5 μM $InsP_3$. The suppressive effect of TBT was significant at 0.1 μM, but not at 25 nM (Fig. 8).

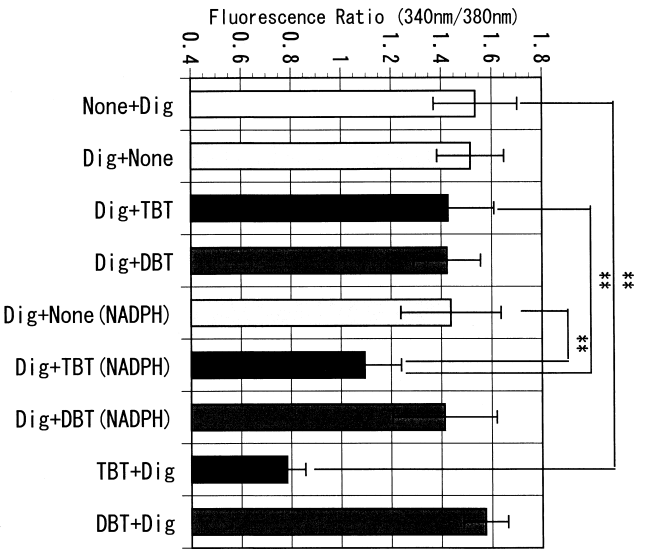

Fig. 6. Effects of TBT- and DBT-treatments on the fura-2 fluorescence ratio in permeabilized cultured hepatocytes, and the effect of NADPH on the organotin-treated cells. Primary cultured hepatocytes were incubated with fura-2/AM for about 2 hr. NADPH emits fluorescence when it is excited at wavelengths of 340 or 380 nm. In this experimental condition, fluorescence intensity of NADPH was usually less than 10% of that of fura-2 in the intracellular organelle. However, the fluorescence of NADPH influenced the fluorescence ratio of fura-2. Thus, we subtracted the fluorescence intensity of 2 μM NADPH from the fluorescence intensity in each image, and then the fluorescence ratio was calculated. None + Dig: Hepatocytes incubated in buffer B for 30 min at 32° and then treated with 15–20 μg/mL of digitonin; Dig + None: Hepatocytes treated with 15–20 μg/mL of digitonin, and then incubated in buffer B for 30 min at 32°; Dig + TBT or Dig + DBT: Hepatocytes treated with 15–20 μg/mL of digitonin and then incubated in KRH containing 4.0 μM TBT or 4.0 μM DBT, respectively, for 30 min at 32°; Dig + None (NADPH): Hepatocytes treated with 15–20 μg/mL of digitonin, and then incubated in buffer B containing 2 μM NADPH for 30 min at 32°; Dig + TBT (NADPH) or Dig + DBT (NADPH): Hepatocytes treated with 15–20 μg/mL of digitonin, and then incubated in buffer B containing 2 μM NADPH and 4.0 μM TBT or 4.0 μM DBT for 30 min at 32°; TBT + Dig or DBT + Dig: Hepatocytes incubated in KRH containing 4.0 μM TBT or 4.0 μM DBT for 30 min at 32°, and then treated with 15–20 μg/mL of digitonin. Results are the means ± SD of 7–14 cells. Statistical analysis was performed by Scheffe's test. A double asterisk (**) indicates $P < 0.01$.

4. Discussion

In our previous study, we reported that 4.0 μM TBT increases intracellular Ca^{2+} concentration, and that this increase is dependent upon the presence of extracellular Ca^{2+} [22]. However, the present experiments clearly show that the increase in cytoplasmic Ca^{2+} concentration was due, at least in part, to the release of Ca^{2+} from the intracellular stores. That TBT releases Ca^{2+} from intracellular stores has already been reported by Chow *et al*. [16] and Oyama *et al*. [30]. Corsini *et al*. [18] also suggested that tributyltin releases Ca^{2+} from intracellular stores in keratinocytes. However, these authors reached their conclusions based upon the measurement of cytoplasmic Ca^{2+} concentration utilizing chemicals that affected the Ca^{2+} stores,

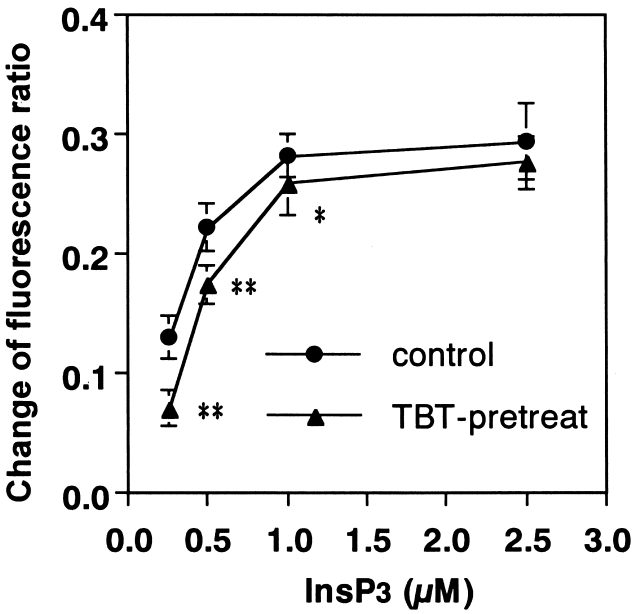

Fig. 7. Suppression of $InsP_3$-induced Ca^{2+} release by TBT-treatment. Hepatocytes were incubated with fura-2/AM for 1.5 hr. The cells were then incubated in buffer A without (control) or with (TBT-pretreated) 0.1 μM TBT for 30 min at 32°. Changes in the fluorescence ratio (340/380 nm) by the addition of $InsP_3$ were measured in each cell. Results are the means ± SD of 6–10 cells. Statistical analysis was performed by Scheffe's test. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs controls.

themselves, rather than by direct measurement of Ca^{2+} in those stores. Our present results showed the depletion of Ca^{2+} from the intracellular stores based upon direct measurement, using digitalized fluorescence microscopy technology.

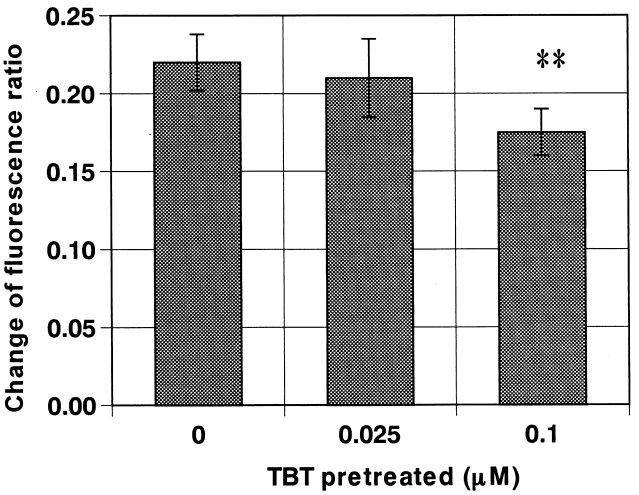

Fig. 8. Concentration-response relationship in the suppression of $InsP_3$-induced Ca^{2+} release by TBT-treatment. Hepatocytes were incubated with fura-2/AM for 1.5 hr. The cells were then incubated in buffer A with TBT for 30 min at 32°. The changes in the fluorescence ratio (340/380 nm) by the addition of 0.5 μM $InsP_3$ were measured in each cell. Results are the means ± SD of 4–10 cells. Statistical analysis was performed by Scheffe's test. Key: (**) $P < 0.01$ vs controls.

We also found that the depletion of Ca^{2+} was not apparent when digitonin-permeabilized hepatocytes were incubated with TBT. There were two possible explanations for this difference in the depleting effects of TBT in intact hepatocytes compared with permeabilized hepatocytes. The first was that the depletion resulted from a decrease in the intracellular content of ATP, upon which the uptake of Ca^{2+} into the stores depends. In our previous paper, we found that 4.0 μM TBT induces cytotoxicity, including loss of ATP [22]; and the present results showed that Ca^{2+} was released from the stores of permeabilized hepatocytes in the absence of ATP. However, the decrease of Ca^{2+} stores in permeabilized cells was much smaller than that in permeabilized hepatocytes preincubated with 4.0 μM TBT. This raised the second possibility—that TBT was metabolized to another active substance that depleted Ca^{2+} from the stores. In fact, we found that the Ca^{2+}-releasing effect of TBT was manifested in the presence of NADPH, which is a cofactor in microsomal mixed-function oxidase systems. It was assumed that the main metabolite of TBT was DBT, and that the debutylation was catalyzed by cytochrome P-450, which generally reacts with TBT in an NADPH-dependent manner [2,9,28,29]. Contrary to our expectations, however, DBT was not the active metabolite, because the effect of DBT on Ca^{2+}-release was very weak. Thus, at present, we are still uncertain as to the identity of the active metabolite. We also cannot rule out the possibility that another cytoplasmic factor(s) also contributes to the depletion of Ca^{2+}. Further studies will be needed to clarify the mechanism of the Ca^{2+}-releasing effect of TBT from the intracellular stores.

In our previous paper, we also reported that pretreatment with a low concentration (0.1 μM) of TBT for 30 min suppressed the Ca^{2+}-responses induced by hormonal stimulations in primary cultured hepatocytes. At this concentration, TBT neither demonstrated cytotoxicity nor decreased $InsP_3$-production. In the present study using a digitonin-permeabilized cell suspension, the increase in Ca^{2+} concentration showing the release of Ca^{2+} from intracellular stores was slightly, but not significantly, suppressed in cells pretreated with 0.1 μM TBT. However, we observed that the release of Ca^{2+} from the stores by $InsP_3$ was decreased significantly by the TBT-pretreatment in the experiments using fura-2-compartmentalized cells. The decrease was more profound in the release by a lower concentration of $InsP_3$, agreeing with the results in the previous report that the suppression of the Ca^{2+} response was more profound when a lower concentration of phenylephrine was used. The results suggest that pretreatment with a low concentration (0.1 μM) of TBT for 30 min suppressed $InsP_3$-induced Ca^{2+} release from the intracellular stores, resulting in the suppression of hormone-induced Ca^{2+}-responses.

What, then, is the mechanism of the suppression of the $InsP_3$-induced Ca^{2+}-release by TBT-pretreatment? $InsP_3$-induced calcium release is known to be regulated by the concentration of $InsP_3$, the cytoplasmic Ca^{2+} concentration

[31–34], and the lumenal Ca^{2+} concentration [35–37]. Under the present experimental conditions, $InsP_3$ concentration and cytoplasmic Ca^{2+} concentration were expected to be the same between the control cells and the TBT-pretreated cells, because we added the same concentration of $InsP_3$ and used the Ca^{2+}-EGTA buffer as the solution to keep the free Ca^{2+} concentration at about 130 nM. Nor was there a difference in the lumenal Ca^{2+} concentration between the control cells and the TBT-pretreated cells in our assay systems using fura-2. It is possible that the dissociation constant of fura-2 to Ca^{2+} was too low to detect the small decrease in the lumenal Ca^{2+} concentration, although we could not detect such a difference in the preliminary experiments using a low-affinity Ca^{2+} fluorescence dye, indo-1-FF. It has been reported that decreases in lumenal Ca^{2+} concentration suppress the opening of the $InsP_3$-receptor channel [35–37]. Therefore, a decrease in the lumenal Ca^{2+} concentration, which could not be detected by the present assay method, may have suppressed the release of Ca^{2+} by $InsP_3$. However, we cannot rule out the other possibility, i.e. that TBT, or its metabolite(s), had a direct effect on the opening of the $InsP_3$-receptor channel. Further studies will be needed to elucidate this mechanism.

In summary, we showed that TBT caused the release of Ca^{2+} from, and depleted Ca^{2+} in, the intracellular stores of hepatocytes. Some part of the depleting effect may have been due to a decrease in the intracellular ATP content. It is also possible, however, that TBT was metabolized to an active form(s) that released Ca^{2+}. In addition, TBT at the low, noncytotoxic concentration of 0.1 μM suppressed the $InsP_3$-induced Ca^{2+} release from the intracellular stores in hepatocytes, resulting in suppression of the Ca^{2+} response induced by hormonal stimulations. In the liver, Ca^{2+} works as a second messenger in the α-adrenergic receptor-mediated effects of epinephrine, such as glycogenolysis and gluconeogenesis [38]. The suppression of such cellular processes may be directly related to functional toxicity, by which the concentration of glucose in the blood does not rise in response to an urgent need. Such toxicity is not lethal, but there may be a sense of exhaustion. Ca^{2+} also seems to act as one of the second messengers for hepatocyte growth factor (HGF) in hepatocytes [39]. It is probable that TBT also suppresses the Ca^{2+} response by HGF, because HGF induces this response via an increase in $InsP_3$ production [40–42]. Therefore, liver regeneration could be suppressed by low concentrations of TBT. Finally, we emphasize that the TBT concentration of 0.1 μM, which here suppressed the $InsP_3$-induced Ca^{2+} release, is lower than, or comparable to, the levels of tri-*n*-butyltin found in marine mammals [2,23–25]. At present, there is no evidence that the incidence of hepatic injury is elevated in marine mammals. However, there is a possibility that accumulation of tri-*n*-butyltin could induce hepatic toxicity in these mammals.

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