Taurolithocholate and taurolithocholate 3-sulphate exert different effects on cytosolic free Ca^{2+} concentration in rat hepatocytes

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Single rat hepatocytes show repetitive oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) when stimulated by agonists acting through the phosphoinositide signalling pathway. We have studied the effect of a natural bile acid, taurolithocholate (TLC), and its sulphated form, taurolithocholate 3-sulphate (TLC-S), on $[Ca^{2+}]_i$ in single isolated rat hepatocytes. Although these bile acids are believed to act through a common mechanism to permeabilize the intracellular Ca^{2+} pool, the $[Ca^{2+}]_i$ responses induced by the two compounds were different. Whereas TLC induced a sustained elevation of $[Ca^{2+}]_i$, TLC-S evoked repetitive $[Ca^{2+}]_i$ oscillations. In addition, we show that ryanodine, which blocks the Ca^{2+}-induced Ca^{2+} release ('CICR') mechanism, blocked TLC-S-induced oscillations in 50% of hepatocytes, but did not affect the TLC-induced rise in $[Ca^{2+}]_i$.

INTRODUCTION

Oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) have been demonstrated in many cell types, including rat hepatocytes, in response to agonists acting through the phosphoinositide signalling pathway (Cobbold et al., 1991; Berridge, 1990). In single aequorin-injected hepatocytes, the oscillations induced by individual agonists display time courses which are characteristic of the receptor species being activated (Woods et al., 1987). The frequency of these oscillations depends on agonist concentration; however, the time course of oscillations induced by a given agonist does not change with agonist dose. We have recently reported that ryanodine, a blocker of the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Meldolesi et al., 1990), has no effect on phenylephrine-induced oscillations, but does block vasopressin-induced oscillations (Cobbold et al., 1991; Sanchez-Bueno and Cobbold, 1993).

The monohydroxylated bile acids, which include taurolithocholate (TLC) and its 3-sulphate (TLC-S), have been shown to increase $[Ca^{2+}]_i$ in populations of rat hepatocytes (Combettes et al., 1988, 1989). In addition, it has been shown that single guinea-pig hepatocytes generate oscillations in Ca^{2+}-dependent K^+ permeability when challenged with TLC-S (Capiod et al., 1991). These bile acids mobilize Ca^{2+} from an internal pool which is sensitive to Ins(1,4,5)P_3 (Combettes et al., 1988, 1989). However, bile acid-mediated Ca^{2+} release is independent of Ins(1,4,5)P_3 production and is believed to be achieved through a direct effect on the permeability of the Ca^{2+} store specifically to Ca^{2+} (Combettes et al., 1988, 1989; Noel et al., 1992).

Here we have investigated the effect of TLC and TLC-S on $[Ca^{2+}]_i$ in aequorin-injected rat hepatocytes, and show that, whereas TLC induced a sustained rise in $[Ca^{2+}]_i$, TLC-S was able to generate $[Ca^{2+}]_i$ oscillations. These oscillations were blocked by ryanodine in 50% of cells, suggesting a role for the CICR mechanism in TLC-S-induced $[Ca^{2+}]_i$ oscillations. The rise in $[Ca^{2+}]_i$ induced by TLC was unaffected by the application of ryanodine.

MATERIALS AND METHODS

Single hepatocytes were isolated from fed male Wistar-strain rats (200–250 g) by collagenase perfusion. The cells were prepared for microinjection with the photoprotein aequorin as described previously (Cobbold and Lee, 1991). Microinjection of aequorin and collection of data were as described by Cobbold and Lee (1991). The experimental medium was Williams Medium E (Flow Laboratories) to which phenylephrine, TLC-S (both from Sigma Chemical Co.) and ryanodine (Calbiochem) were added. TLC (Sigma Chemical Co.) was dissolved in dimethyl sulphoxide to give a 10 mM stock. Portions of this were added to Williams Medium E to give the required concentration.

RESULTS

The application of the bile acids TLC and TLC-S to aequorin-injected rat hepatocytes produced different effects on $[Ca^{2+}]_i$. In 16/18 hepatocytes, TLC at a concentration of 200 μM produced an increase in $[Ca^{2+}]_i$, which rose over a period of 5–10 min from the resting level of approx. 200 nM to a maximum of approx. 600 nM (Figure 1a). There was a delay of between 5 and 20 min before the onset of the rise (see Figure 1a). The effect of TLC was not reversible, as illustrated in Figure 1(a); the $[Ca^{2+}]_i$ remained elevated 20 min after removal of TLC. At much lower concentrations (5–10 μM), TLC induced a rise which showed evidence of oscillatory behaviour, but the oscillations were irregular in both frequency and duration and were superimposed on an elevated background (Figure 1b; 4/7 cells). TLC never induced discrete oscillations comparable with those induced by Ins(1,4,5)P_3-dependent agonists. Control applications of dimethyl sulphoxide, the solvent for TLC, did not affect the $[Ca^{2+}]_i$ in 5/5 hepatocytes. In contrast, TLC-S in the range 200–400 μM induced $[Ca^{2+}]_i$ oscillations in 23/24 hepatocytes. These oscillations were similar to those induced by Ins(1,4,5)P_3-dependent agonists, rising rapidly from the resting $[Ca^{2+}]_i$ of $\sim$ 200 nM to a peak of $\sim$ 600 nM, followed by a fall back to the resting level (Figure 2).

Abbreviations used: CICR, Ca^{2+}-induced Ca^{2+} release; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; TLC, taurolithocholate; TLC-S, taurolithocholate 3-sulphate.

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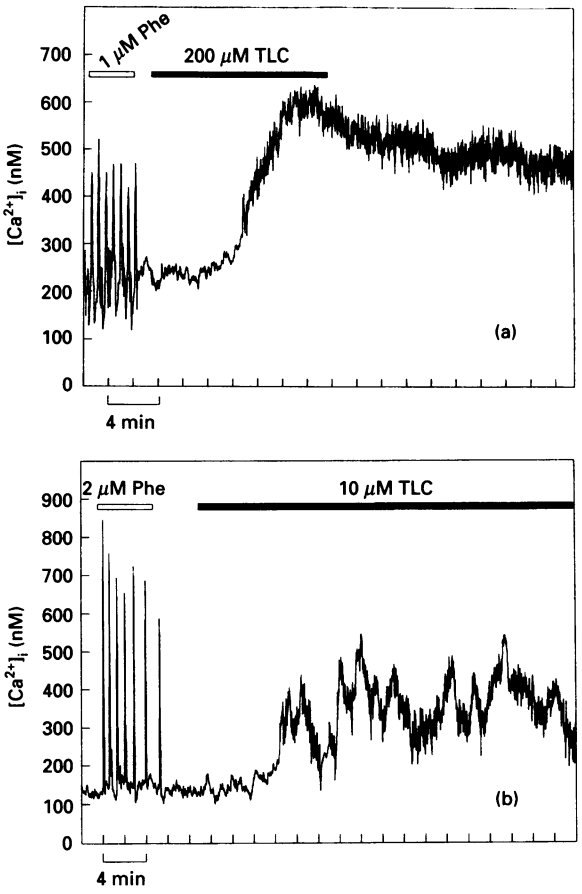

Figure 1 Rises in $[Ca^{2+}]_i$ induced in aequorin-injected hepatocytes by TLC

(**a**) A single aequorin-injected hepatocyte stimulated with 1 μM phenylephrine (Phe) produced oscillations in $[Ca^{2+}]_i$. 200 μM TLC induced a slow rise in $[Ca^{2+}]_i$ to a peak of ~ 600 nM. The effect was not reversible; the $[Ca^{2+}]_i$ remained elevated 20 min after removal of the TLC. (**b**) At a lower concentration (10 μM), TLC induced oscillatory rises in $[Ca^{2+}]_i$, although these were irregular, and not comparable with those induced by phenylephrine in the same cell. Time constants for both: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

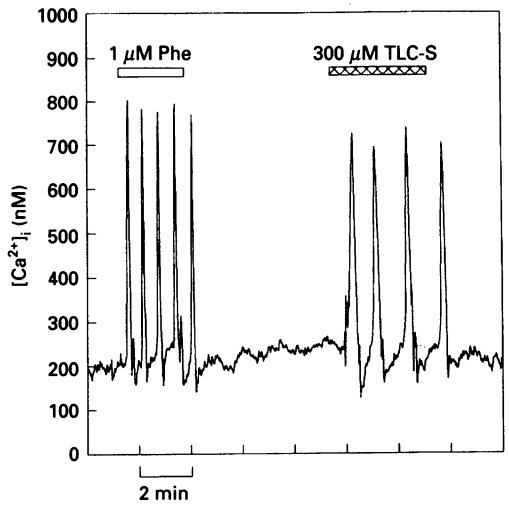

Figure 2 Oscillations in $[Ca^{2+}]_i$ induced by TLC-S displaying the short latency period and prompt reversal of the effect when TLC-S was removed

An aequorin-injected hepatocyte was stimulated with 1 μM phenylephrine (Phe) and produced oscillations in $[Ca^{2+}]_i$. Subsequent stimulation with 300 μM TLC-S produced discrete $[Ca^{2+}]_i$ oscillations after a latency period of less than 1 min. When TLC-S was washed off, the oscillations promptly stopped. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

As with Ins(1,4,5)P_3-mediated oscillations, the latency period between agonist addition and the onset of the cellular response was short (0.5–3 min), and was comparable with the period between oscillations. The oscillations promptly stopped when TLC-S was withdrawn (Figure 2). However, a given Ins(1,4,5)P_3-dependent agonist, with the exception of ATP (Dixon et al., 1990), induces oscillations of consistent duration in the same cell and also between cells (Woods et al., 1987). In contrast, TLC-S induced oscillations which varied in duration from cell to cell, reminiscent of the variable responses of cells to ATP. Of the 23 hepatocytes responding to TLC-S, 9 produced oscillations of short duration (~ 10–18 s; Figure 3a). In a further 4 hepatocytes the transients induced by TLC-S were prolonged with slow falling phases and overall durations in the range ~ 30–120 s (Figure 3b). The remaining 10 hepatocytes displayed oscillations with a slower rate of rise of $[Ca^{2+}]_i$ from the resting level to the peak, leading to oscillations with almost symmetrical profiles and durations of ~ 18–30 s. These different patterns do not reflect variability in the condition of the hepatocytes; cells were routinely checked for a characteristic response to phenylephrine before addition of TLC-S. Figure 4 compares the oscillations induced by TLC-S and phenylephrine in the same cell, showing the slower rate of rise of $[Ca^{2+}]_i$ for TLC-S-induced oscillations. TLC-S is unusual in inducing oscillations with a slower rate of rise, as this parameter has previously been shown to remain constant for oscillations induced by all Ins(1,4,5)P_3-mediated agonists (Woods et al., 1987).

When applied in combination with phenylephrine, TLC irreversibly blocked the $[Ca^{2+}]_i$ oscillations induced by phenylephrine (Figure 5; 9/9 cells). TLC was effective at concentrations as low as 0.5 μM, which alone did not affect $[Ca^{2+}]_i$. Furthermore, after application of TLC at any concentration, no further response was elicited by Ins(1,4,5)P_3-dependent agonists. In contrast, application of TLC-S at concentrations up to 500 μM did not inhibit subsequent cellular responses.

It has been shown that TLC-S releases Ca^{2+} from internal non-mitochondrial stores independently of Ins(1,4,5)P_3 (Combettes et al., 1988, 1989). We therefore investigated the effect of ryanodine, which blocks CICR, on the oscillations induced by TLC-S. The application of 10–20 μM ryanodine inhibited oscillations induced by TLC-S in 6/12 hepatocytes (Figure 6). Of the 6 cells in which the oscillations were not blocked, two cells showed a decrease in frequency of TLC-S-induced oscillations. In contrast, ryanodine had no effect on the TLC-induced changes in $[Ca^{2+}]_i$ in 5/5 hepatocytes (results not shown).

DISCUSSION

The monohydroxylated bile acids, including TLC and TLC-S, inhibit the secretion of bile from rat liver (Javitt, 1966). This effect has been attributed to their ability to raise $[Ca^{2+}]_i$, as demonstrated in populations of rat hepatocytes (Combettes et al., 1988, 1989). In addition, TLC-S, at concentrations in the range used here, has been shown to induce oscillations in Ca^{2+}-dependent K^+ permeability in guinea-pig hepatocytes (Capiod et al., 1991; Noel et al., 1992). Calcium is released by TLC and TLC-S from the Ins(1,4,5)P_3-sensitive store, but the release is believed to be independent of Ins(1,4,5)P_3 production. Application of neomycin, which binds Ins(1,4,5)P_3, or heparin, which competes for

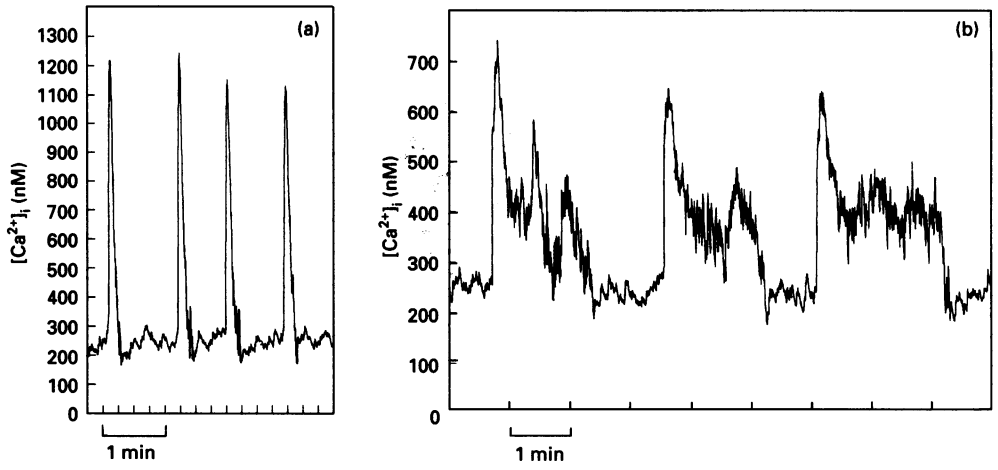

Figure 3 Variability in the duration of $[Ca^{2+}]_i$ oscillations induced by TLC-S

(**a**) $[Ca^{2+}]_i$ oscillations of short duration induced in a single hepatocyte by 300 μM TLC-S. Similar oscillations were recorded in 9/24 hepatocytes. (**b**) $[Ca^{2+}]_i$ oscillations induced in a single hepatocyte by 400 μM TLC-S, showing prolonged falling phases. Similar oscillations were recorded in 4/24 hepatocytes. Time constants: for resting $[Ca^{2+}]_i$, 4 s; for oscillations, (**a**) 0.4 s, (**b**) 0.8 s.

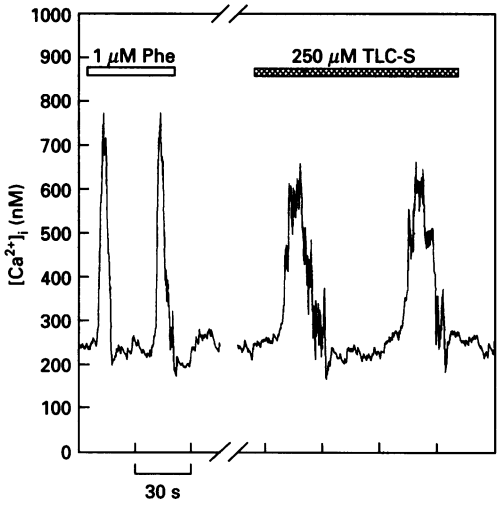

Figure 4 Detailed profiles of phenylephrine- and TLC-S-induced $[Ca^{2+}]_i$ oscillations showing the difference in the rising phase

The oscillations were recorded from the same hepatocyte in response to 1 μM phenylephrine (Phe) and 250 μM TLC-S, and show the slower rate of rise of $[Ca^{2+}]_i$ for TLC-S-induced oscillations, as seen in 10/24 hepatocytes. Time constants: for resting $[Ca^{2+}]_i$, 4 s; for oscillations, 0.4 s.

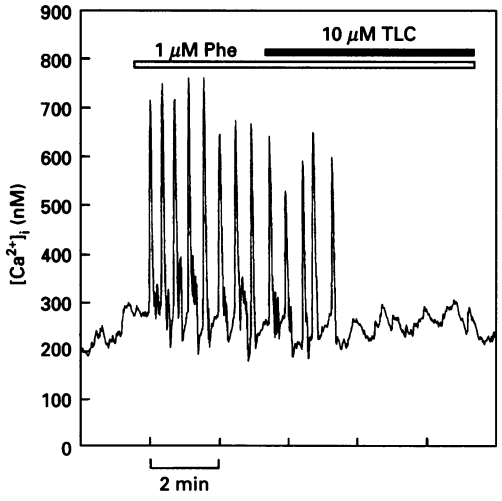

Figure 5 Inhibition of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by application of TLC

TLC (10 μM) was co-applied to an hepatocyte producing oscillations in response to 1 μM phenylephrine (Phe). This treatment rapidly blocked the oscillations. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

the Ins(1,4,5)P_3-binding site, to saponin-treated hepatocytes did not affect Ca^{2+} release by TLC, but inhibited the release by Ins(1,4,5)P_3 (Combettes et al., 1989). In addition, internal perfusion of single guinea-pig hepatocytes with an antibody to PtdIns(4,5)P_2 did not affect the oscillations in Ca^{2+}-dependent K^+ permeability induced by TLC-S, but blocked those induced by noradrenaline (Noel et al., 1992). Both TLC and TLC-S are believed by these groups to effect Ca^{2+} release through a direct and specific permeabilizing action on the intracellular Ca^{2+} store, without affecting the permeability of plasmalemmal and mitochondrial membranes, through a mechanism which is not yet understood (Combettes et al., 1988, 1989). However, the data reported here show that in single rat hepatocytes the effects of TLC and TLC-S on $[Ca^{2+}]_i$ are very different. Whereas TLC induced a sustained rise in $[Ca^{2+}]_i$, TLC-S induced $[Ca^{2+}]_i$ oscillations which are remarkedly similar to those generated by Ins(1,4,5)P_3-dependent agonists (Woods et al., 1986; Cobbold et al., 1991). TLC and TLC-S have different biological effects: TLC produces cholestasis in a dose-dependent manner (Javitt, 1966), a phenomenon that is not observed after administration of the sulphated form (Yousef et al., 1981). This difference has previously been attributed to a slower rate of uptake of TLC-S into cells because of its increased water-solubility, or differences in the binding of TLC-S to intracellular proteins, effectively decreasing

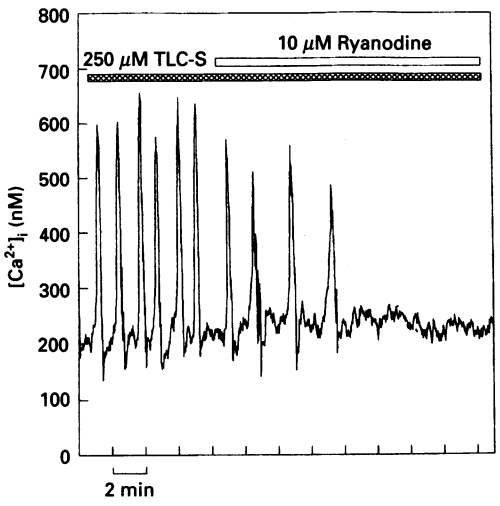

Figure 6 Inhibition of TLC-S-induced $[Ca^{2+}]_i$ oscillations by application of ryanodine

Ryanodine (10 μM) was applied to an hepatocyte producing oscillations in response to 250 μM TLC-S. This treatment blocked TLC-S-induced oscillations in 6/12 hepatocytes. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

the concentration of free bile acid (Combettes et al, 1989). Alternatively, the different biological effects may reflect the differences in the Ca^{2+} responses of hepatocytes to TLC and TLC-S which are reported here. These data indicate that TLC irreversibly disrupts the cell's oscillatory mechanism, blocking phenylephrine-induced oscillations and preventing the cell from responding to subsequent stimulation by Ca^{2+}-mobilizing agonists. In contrast, the effect of TLC-S was readily reversible and did not interfere with the subsequent production of oscillations, in accordance with the findings of Capiod et al. (1991). It is difficult to reconcile the disparate effects of TLC and TLC-S on $[Ca^{2+}]_i$ with a single mode of action such as membrane permeabilization.

The oscillations induced by TLC-S varied from cell to cell, ranging from oscillations of short duration (Figure 3a) to much longer duration (Figure 3b). Capiod et al. (1991) described two types of oscillation in Ca^{2+}-dependent K^+ permeability in response to TLC-S. Type I oscillations occur at high frequency, and are superimposed on an elevated $[Ca^{2+}]_i$. Type II oscillations occur at much lower frequency, and are separated by periods where $[Ca^{2+}]_i$ is at the resting level. The different oscillations reported here were all separated by periods where $[Ca^{2+}]_i$ fell to the resting level and therefore, according to this classification, are all type II oscillations. No responses corresponding to the type I oscillations described by Capiod et al. (1991) were recorded. However, the aequorin measurements described here are measurements of the average $[Ca^{2+}]_i$ changes throughout the cell. Those reported by Capiod et al. (1991) are indirect recordings of changes in sub-plasmalemmal $[Ca^{2+}]_i$, which may not be detected by aequorin. The differences in the nature of the oscillations may therefore be the result of the different techniques for monitoring $[Ca^{2+}]_i$ which were employed.

The rate of rise of $[Ca^{2+}]_i$ from the resting level to the peak of the oscillation does not differ significantly for oscillations evoked by different Ins(1,4,5)P_3-dependent agonists (Woods et al., 1987). Capiod et al. (1991) reported that the rate of rise of oscillations in Ca^{2+}-dependent K^+ permeability evoked by both noradrenaline and TLC-S was the same. Approx. 43% of the hepatocytes produced oscillations in response to TLC-S which had a much slower rate of rise than the oscillations induced by phenylephrine in the same cell, indicating that in these cells the two agonists were not acting through a common mechanism.

As TLC-S oscillations are believed to be independent of Ins(1,4,5)P_3 production, the role of CICR mediated by ryanodine receptors was investigated. High-affinity binding sites for ryanodine, which blocks the CICR channel in the open state, have been found in rat liver microsomes. These ryanodine-binding sites are distinct from Ins(1,4,5)P_3-binding sites (Shoshan-Barmatz et al., 1990). We have recently shown that, although ryanodine does not affect phenylephrine-induced $[Ca^{2+}]_i$ oscillations, it blocks vasopressin-induced oscillations in a proportion of cells, suggesting a role for CICR, or at least the ryanodine receptor in these oscillations (Sanchez-Bueno and Cobbold, 1993). TLC-S-induced $[Ca^{2+}]_i$ oscillations were blocked in the same proportion of cells as vasopressin-induced oscillations, indicating a similar involvement of the CICR mechanism in the generation of these oscillations. It is conceivable that the oscillations with a slower rate of rise were generated through CICR mediated by ryanodine receptors, without involving Ins(1,4,5)P_3 receptors, in agreement with the mechanism proposed by Noel et al. (1992).

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