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Taurolithocholate-induced Ca^{2+} release is inhibited by phorbol esters in isolated hepatocytes

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The monohydroxy bile acid taurolithocholate (TLC) causes a rapid and transient increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in suspensions of rat hepatocytes similar to that elicited by the $InsP_3$-dependent hormone vasopressin. The effect of the bile acid is due to a mobilization of Ca^{2+}, independent of $InsP_3$, from the endoplasmic reticulum (ER). Short-term preincubation of cells with the phorbol ester 4β-phorbol 12β-myristate 13α-acetate (PMA), which activates protein kinase C (PKC), blocked the increase in $[Ca^{2+}]_i$ induced by TLC, but did not alter that mediated by vasopressin. We obtained the following results, indicating that the effect of PMA is mediated by the activation of PKC. (1) Phorbol esters were effective over a concentration range where they activate PKC (IC_{50} = 0.5 nM); (2) phorbol esters that do not activate PKC did not inhibit the effects of TLC; (3) the permeant analogue oleoylacetylglycerol mimicked the inhibitory effect of PMA; (4) lastly, the inhibition of the TLC-induced Ca^{2+} mobilization by phorbol esters was partially prevented by preincubating the cells with the PKC inhibitors H7 and AMG-C_{16}. Preincubating hepatocytes with PMA had no effect on the cell uptake of labelled TLC, indicating that the phorbol ester does not interfere with the transport system responsible for the accumulation of bile acids. In saponin-treated liver cells, PMA added before or after permeabilization failed to abolish TLC-induced Ca^{2+} release from the ER. The possibility is discussed that PMA, via PKC activation, may alter the intracellular binding or the transfer of bile acids in the liver.

INTRODUCTION

In vivo, most of the circulating bile acids (trihydroxy) are concentrated by the hepatocyte, translocated via binding proteins and Golgi-derived vesicles to the biliary pole of the cell, where they are actively secreted. This transport generates mostly native bile (Hofmann, 1988; Erlinger, 1990; Nathanson & Boyer, 1991). Others, such as the monohydroxy bile acid taurolithocholate, though accumulated similarly (Hofmann, 1988; Combettes *et al.*, 1990), dramatically inhibit bile secretion (Javitt, 1975). An unexpected characteristic of these inhibitory bile acids is that they induce a rapid and transient increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat liver cells and in a kidney cell line (Anwer *et al.*, 1988; Combettes *et al.*; 1988*a*, Montrose *et al.*, 1988). In the liver, the endoplasmic reticulum (ER) has been identified as the target of the action of monohydroxy bile acids on intracellular Ca^{2+} (Combettes *et al.*, 1988*b*, 1989). Indeed, we have found that monohydroxy bile acids mobilize Ca^{2+} from the same internal Ca^{2+} pool as does the $InsP_3$-dependent hormone vasopressin in intact cells (Combettes *et al.*, 1988*a*, 1990) and as does the messenger $InsP_3$ in saponin treated cells (Combettes *et al.*, 1988*b*, 1989). Despite having a common target, $InsP_3$-linked hormones and bile acids appear to release Ca^{2+} via different mechanisms. Thus the action of monohydroxy bile acids is apparently independent of the phospholipase C/$InsP_3$ pathway; rather, these molecules act selectively and directly on the ER membrane (Combettes *et al.*, 1988*b*, 1989; Capiod *et al.*, 1991), differently from a Ca^{2+}-ionophoretic effect (for recent references, see Zimniak *et al.*, 1991). Another unexpected characteristic is that the monohydroxy bile acids do not increase $[Ca^{2+}]_i$ in other cell types, such as human platelets or neuronal cells, because these cells do not possess the carrier for their accumulation. Bile acids do release Ca^{2+} from the ER of such cells, provided that the plasma membrane has been previously permeabilized by saponin (Coquil *et al.*, 1991).

While studying the mechanism of action of these molecules, we were surprised to observe that activation of protein kinase C (PKC) inhibited the taurolithocholate (TLC)-induced Ca^{2+} increase in intact hepatocytes, an effect which may be relevant to the recent observation that PKC inhibits bile secretion in the liver (Corasanti *et al.*, 1989). In the present study, we attempted to characterize how PKC activation inhibits the movement of Ca^{2+} generated by the bile acid. To examine the effects of PKC on the TLC-induced $[Ca^{2+}]_i$ increase in isolated rat liver cells, we employed diacylglycerol (DAG) and tumour-promoting phorbol esters. The latter are structural analogues of DAG, which have been previously used as molecular probes to isolate functionally and to study PKC (Nishizuka, 1986).

MATERIALS AND METHODS

Materials

Quin2 and quin2/AM were obtained from Lancaster Synthesis (Morecambe, Lancs., U.K.), 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG-C_{16}) was from Bachem A.G. (Bubendorf, Switzerland), ionomycin was from Calbiochem, collagenase was from Boehringher, amino acids (with L-glutamine) and vitamins were from GIBCO, dimethyl sulphoxide (DMSO) was from Merck, Ins(1,4,5)P_3 was from Amersham Corp., [^{14}C]TLC was from Mallinckrodt Chemical Corp. (St. Louis, MO, U.S.A.), and [^{32}P]P_i was from Oris (Gif/Yvette, France). All other chemicals were purchased from Sigma and were of the highest grade commercially available.

Preparation and incubation conditions

Isolated hepatocytes were prepared by collagenase perfusion

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; TLC, taurolithocholic acid; PKC, protein kinase C; DAG, diacylglycerol; PMA, 4β-phorbol 12β-myristate 13α-acetate; αPDD, 4α-phorbol 12β,13β-didecanoate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; AMG-C_{16}, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol; quin2, 2-{[2-bis(carboxymethyl)-amino-5-methylphenoxyl]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline; quin2/AM, quin2 tetra-acetoxymethyl ester; ER, endoplasmic reticulum; DMSO, dimethyl sulphoxide; OAG, oleoylacetylglycerol.

from fed female Wistar rats as previously described (Binet *et al.*, 1985). Briefly, rat hepatocytes were maintained (2×10^6 cells/ml) in Eagle's medium, consisting of 116 mM-NaCl, 5.4 mM-KCl, 12 mM-$CaCl_2$, 0.8 mM-$MgCl_2$, 0.92 mM-NaH_2PO_4, 25 mM-$NaHCO_3$, 15 mg of gelatin/ml, vitamins and amino acids, and 5.6 mM-glucose. It was gassed with O_2/CO_2 (19:1) (pH 7.4) at 37 °C. Cell viability, estimated by Trypan Blue exclusion, was always greater than 96% and remained stable for 4–5 h.

Quin2 loading of cells

Incubation conditions for loading cells with quin2 and measuring changes in cellular free Ca^{2+} concentration have been described previously (Binet *et al.*, 1985): cells (2×10^{10} cells/ml) were loaded with 50 μM-quin2/AM for 150 s, then washed twice and transferred (0.5×10^6 cells/ml) into the cuvette of the spectrofluorimeter at 37 °C, under continuous agitation and gassing with O_2/CO_2 (19:1). $[Ca^{2+}]_i$ was measured, calibrated and corrected for autofluorescence as reported by Binet *et al.* (1985).

Influx of labelled bile acids

Incubation conditions for the uptake of bile acids have been described previously (Combettes *et al.*, 1990). The uptake was initiated by incubating cells (2×10^6/ml) at 37 °C in Eagle's medium supplemented with 100 μM-TLC and traces of [^{14}C]TLC (2 μCi/ml). Cells were sampled (150 μl) at 5, 15, 30, 60, 90 and 120 s after addition of the labelled bile acids. Each sample was immediately transferred to Eppendorf tubes containing 1 ml of an ice-cold 'washing solution' consisting of 154 mM-NaCl, 2 mM-EGTA, 2% BSA and 200 μl of dibutyl phthalate ($d = 1.045$), pH 7.4. As reported previously (Combettes *et al.*, 1990), BSA was used to decrease the non-specific binding and contaminating extracellular bile acids. The latter amounted to 0.37 ± 0.09 nmol/mg of protein ($n = 4$) and was unaltered by 4β-phorbol 12β-myristate 13α-acetate (PMA). This value was small as compared with the intracellular [^{14}C]TLC uptake by liver cells measured at 120 s (approx. 5 nmol/mg of protein; see Fig. 4). The mixture was then centrifuged at 9000 *g* for 1 min. The cell pellets were dispersed in water. Samples (100 μl) were counted for radioactivity in a scintillation spectrometer. TLC influx was calculated from the rate of the initial phase of uptake, i.e. over 2 min.

Ca^{2+} release from saponin-treated cells

Hepatocytes (2.5×10^6 cells/ml) were incubated in a cytosol-like medium and permeabilized by saponin (50 μg/ml) as reported by Burgess *et al.* (1984). The medium consisted of 20 mM-NaCl, 100 mM-KCl, 5 mM-$MgCl_2$, 0.96 mM-NaH_2PO_4 and 25 mM-Hepes (pH 7.15, with KOH) at 37 °C. The medium also contained 1.5 mM-ATP, 5 mM-phosphocreatine, 5 units of creatine kinase/ml and 10–20 μM-quin2. The concentration of free Ca^{2+} and the amount of Ca^{2+} released by the cells were calculated as reported in Binet *et al.* (1985).

PMA-mediated phosphorylation in intact and permeabilized cells

Rat hepatocytes (6×10^6 cells/ml) were labelled with 0.3 mCi of [^{32}P]P_i/ml in Eagle's medium for 1 h at 37 °C under continuous agitation and gassing with O_2/CO_2 (19:1). Then cell samples (250 μl) were centrifuged and washed at 50 *g* for 2 min. Hepatocyte pellets were resuspended (2.5×10^6 cells/ml) either in Eagle's medium or in the cytosol-like medium (see above). PMA (100 nM) was added for 2 min before or after the addition of saponin (50 μg/ml). Cells were solubilized in SDS sample buffer, containing 3% SDS, 30 mM-NaF, 1 mM-β-mercaptoethanol and 62 mM-Tris/HCl, pH 6.8, and heated to 100 °C for 3 min before electrophoresis. SDS/PAGE was performed in 10% (w/v) acrylamide gels as described by Laemmli (1970). The gels were stained with Coomassie Blue, destained, dried and exposed to Amersham X-ray film for autoradiography at −80 °C for 48 h.

Ca^{2+} contamination and optical interferences of bile acids with quin2

No Ca^{2+} contamination by bile acids or other molecules used here was detected by atomic-absorption spectroscopy or quin2 fluorescence. In addition, consistent with our previous observations (Combettes *et al.*, 1989), the different agents employed did not alter the optical (absorbance and fluorescence emission) and binding properties (K_D and maximal binding to Ca^{2+}) of free quin2 added to saline solutions. AMG-C_{16} and H7, at 100 μM in DMSO, caused a slight artifactual fluorescence quenching, but the Ca^{2+}-dependent fluorescence response, calibrated after the cells had been permeabilized by saponin by excess of Ca^{2+} and EGTA (Binet *et al.*, 1985), was not affected by the presence of these agents (Table 1).

Presentation of data

Typical Ca^{2+} traces are reported in Figs. 1, 3 and 5. They are representative of at least three similar ones obtained on different cell preparations. The other results are means ± S.E.M. of the numbers of independent experiments indicated. Statistical significance was calculated by Student's *t* test.

Determination of protein content

Protein was determined by the method of Lowry *et al.* (1951). BSA was used as the protein standard.

RESULTS

Effect of PMA on vasopressin- and TLC-induced $[Ca^{2+}]_i$ rise

Previous studies have shown that monohydroxy bile acids such as TLC, TLC sulphate and lithocholate, induce a rapid, large and transient increase in cytosolic free Ca^{2+} resulting from a mobilization of Ca^{2+} from the ER (Combettes *et al.*, 1988*a*,*b*, 1989). The effect occurs with an EC_{50} of about 20 μM (Combettes *et al.*, 1988*a*; Anwer *et al.*, 1988). Fig. 1 shows that the addition of a maximal concentration of TLC (100 μM) induced, as does the InsP_3-dependent hormone vasopressin (10 nM) (Exton, 1988), a $[Ca^{2+}]_i$ increase from 200 nM to approx. 800 nM within 10–20 s.

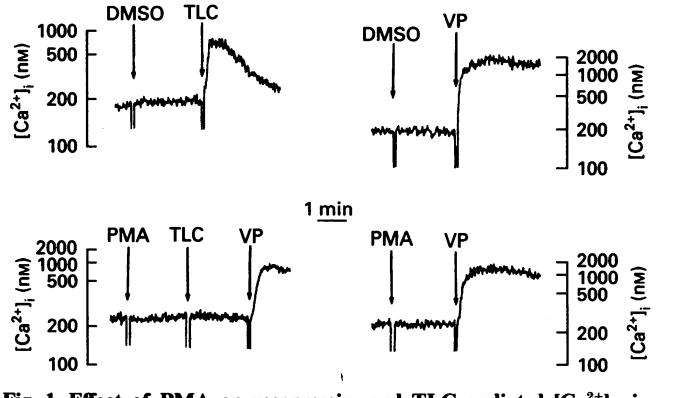

Fig. 1. Effect of PMA on vasopressin- and TLC-mediated $[Ca^{2+}]_i$ rises in hepatocytes loaded with quin2

Cells were loaded with quin2 and $[Ca^{2+}]_i$ was measured as described in the Materials and methods section. Hepatocytes were incubated in an Eagle's medium containing 1.2 mM-Ca^{2+}. TLC and vasopressin (VP), at maximal concentrations (100 μM and 10 nM respectively), or the same volumes (5 μl) of their respective solvents (water or DMSO), were added 2 min after PMA (PMA, 100 nM).

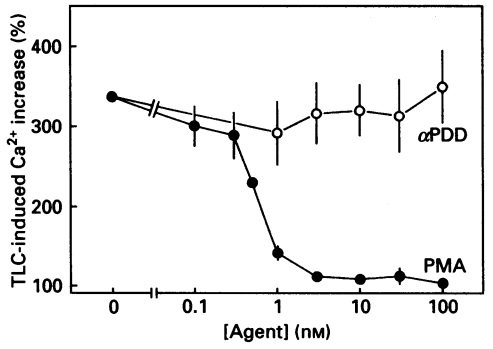

Fig. 2. Effect of PMA and αPDD on $[Ca^{2+}]_i$ rise induced by TLC in intact cells

TLC (100 μM) was added 2 min after either PMA (●) or αPDD (○) at the concentrations indicated (log scale). Results are expressed as the percentage of the control $[Ca^{2+}]_i$ and are means ± S.E.M. of 4–12 experiments.

Table 1. Effects of various regulators of PKC activity on TLC-mediated $[Ca^{2+}]_i$ rises

PMA (1 or 3 nM) was added 2 min before TLC (100 μM), and AMG-C_{16} (100 μM) and H7 (100 μM) were added 5 min before PMA or TLC. The $[Ca^{2+}]_i$ rise is expressed as the percentage of the initial $[Ca^{2+}]_i$ (without agent), which was 210 ± 15 nM ($n = 12$). Agents which affect PKC activity (PMA, AMG-C_{16} or H7) had no effect on basal $[Ca^{2+}]_i$. Data are means ± S.E.M. of the numbers of experiments indicated in parentheses: *significantly different from paired PMA-treated cells ($P < 0.05$).

Agents	Resting $[Ca^{2+}]_i$ (% of control)	TLC-mediated $[Ca^{2+}]_i$ increase (% of control)
Water	100 ± 2 (10)	
DMSO	98 ± 0.5 (12)	365 ± 40 (12)
PMA (1 nM)	106 ± 4 (6)	130 ± 7 (4)
PMA (3 nM)	107 ± 3 (7)	111 ± 6 (7)
AMG-C_{16}	103 ± 2 (4)	310 ± 32 (4)
H7	104 ± 3 (4)	363 ± 54 (4)
PMA (1 nM) + AMG-C_{16}	101 ± 1 (4)	192 ± 23 (4)*
PMA (1 nM) + H7	104 ± 2 (5)	220 ± 34 (5)*
PMA (3 nM) + AMG-C_{16}	110 ± 10 (6)	183 ± 19 (6)*
PMA (3 nM) + H7	100 ± 1 (7)	155 ± 11 (7)*

In contrast with the transient nature of the TLC-mediated $[Ca^{2+}]_i$ rise, the $[Ca^{2+}]_i$ increase arising from the activation of vasopressin receptors was more sustained (compare the typical traces illustrated in Fig. 1). The longer duration of the increase has been ascribed to the fact that $InsP_3$-dependent hormones, in addition to their mobilizing action on the internal Ca^{2+} pool, elicit a sustained influx of extracellular Ca^{2+} (Poggioli *et al.*, 1985). Consistent with the data reported by Cooper *et al.* (1985) and Hoek *et al.* (1988), addition of 100 nM-PMA did not induce any change in the resting $[Ca^{2+}]_i$ (Fig. 1). Nevertheless, Fig. 1 shows that Ca^{2+} mobilization by TLC was almost completely abolished by pretreating the cells for 2 min with 100 nM-PMA. Under the same experimental conditions, PMA had no effect on the Ca^{2+} mobilization by vasopressin (Fig. 1), as previously reported by Lynch *et al.* (1985). Moreover, whereas the Ca^{2+}-mobilizing action of TLC was totally inhibited by 100 nM-PMA, the subsequent addition of vasopressin (10 nM) to the same cells elicited the usual increase in $[Ca^{2+}]_i$ (Fig. 1). That PMA altered neither the basal level of $[Ca^{2+}]_i$ nor the hormone-mediated rise in $[Ca^{2+}]_i$ in intact liver cells is consistent with the view that the PKC activation affects neither passive and active Ca^{2+} fluxes in resting cells nor the ability of the ER to concentrate and release Ca^{2+} in response to vasopressin.

The inhibition of the TLC-induced $[Ca^{2+}]_i$ rise by PMA was time- and dose-dependent. Within 1 min, a marked but incomplete inhibition was observed (results not shown), and 2 min was sufficient for a maximal effect. Fig. 2 shows the dose–response curve for the action of PMA. A concentration as low as 3 nM completely abolished the effect of the bile acid, and half-maximal inhibition occurred with approx. 0.5 nM-PMA, a sensitivity very similar to that reported for PMA-induced PKC activation in a number of cell types, including hepatocytes [Cooper *et al.* (1985); Lynch *et al.* (1985); Corvera *et al.* (1986); see Nishizuka (1986) and Exton (1988) for reviews]. In contrast, 4α-phorbol 12β,13β-didecanoate (αPDD), a phorbol derivative that does not activate PKC (Castagna *et al.*, 1982), did not inhibit the TLC-mediated $[Ca^{2+}]_i$ rise. Indeed, as shown in Fig. 2, the same concentrations of αPDD (1–100 nM) applied for the same time (2 min) had not effect on the response of cells to TLC. The inability of αPDD to affect the TLC-induced Ca^{2+} response suggests that the inhibitory effect of PMA is mediated via the activation of PKC. More evidence on this point is provided by the data shown in Fig. 3(*a*). The synthetic DAG oleoylacetylglycerol (OAG), which activates PKC, markedly inhibited

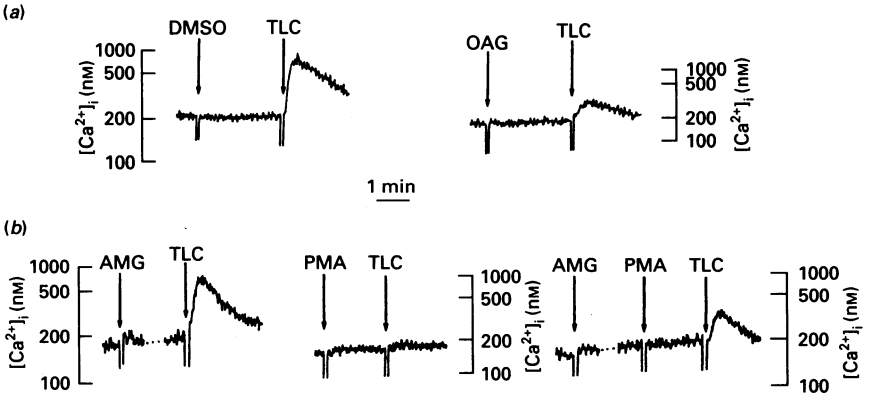

Fig. 3. Effects of PKC activators and inhibitors on TLC-mediated $[Ca^{2+}]_i$ rises in intact cells

(a) OAG (10 μM in DMSO) was added 2 min before TLC (100 μM). (*b*) AMG-C_{16} (AMG; 100 μM) was added 5 min before PMA (3 nM) or TLC (100 μM). For technical convenience, the Ca^{2+} traces were interrupted. Gaps represent 4 min.

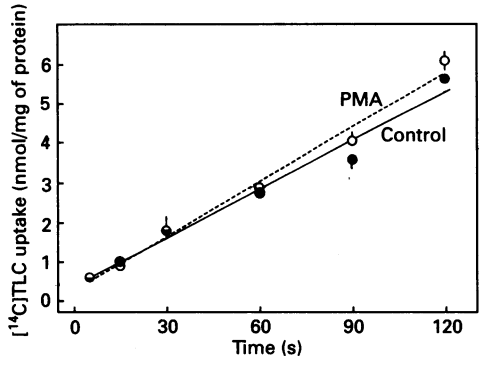

Fig. 4. Effect of PMA on time courses of [^{14}C]TLC uptake by isolated rat liver cells

Uptake was initiated by adding 100 μM-[^{14}C]TLC, without (●) or with MPA (○; 100 nM, 2 min before), at zero time. Results are means ± S.E.M. of 6 experiments.

the TLC-induced $[Ca^{2+}]_i$ cell response. However, in accordance with previous studies by Cooper *et al.* (1985) on the efficiency of OAG in activating PKC in the liver, relatively high concentrations (10 μM) of the DAG analogue were required to produce this effect.

Effects of PKC inhibitors on PMA-mediated inhibition of the TLC-induced $[Ca^{2+}]_i$ increase

A further indication of the involvement of PKC in the coupling mechanism is the observation of a reversibility by PKC inhibitors, as shown in Fig. 3(*b*) and Table 1. In these experiments, two different PKC inhibitors were used for their ability to prevent the effect of PMA on the TLC-induced $[Ca^{2+}]_i$ increase, i.e. H7 and AMG-C_{16}. As shown in Table 1, the inhibition by PMA could be partially prevented by preincubating hepatocytes for 5 min with 100 μM-H7, a protein kinase inhibitor with some specificity for PKC (Hidaka *et al.*, 1984). Similarly, AMG-C_{16} (100 μM), a new PKC inhibitor (Kramer *et al.*, 1989), partially prevented the effect of PMA on the TLC-induced $[Ca^{2+}]_i$ increase (Fig. 3*b* and Table 1). The inhibition by the two PKC antagonists was never complete, perhaps owing to their lack of specificity toward PKC (Huang, 1989). The addition of H7 or AMG-C_{16} alone, i.e. in the absence of PMA, affected neither the TLC-induced transient $[Ca^{2+}]_i$ rise nor the resting $[Ca^{2+}]_i$ (Fig. 3 and Table 1).

Effect of PMA on [^{14}C]TLC uptake

A question that is raised by the above experiments is which step, from the uptake of extracellular TLC to the mobilization of Ca^{2+} from the internal pool, is altered by the activation of PKC. One possibility is a PMA-mediated inhibition of the plasma-membrane transport system which concentrates TLC in the liver cell (Hofmann, 1988). To examine this possibility, we investigated the ability of PMA to alter the uptake of labelled TLC by rat hepatocytes. The uptake of TLC was measured between 5 and 120 s, a period of time during which the uptake represents adequately the unidirectional influx of TLC mediated by the bile acid carrier present in the liver plasma membrane (Combettes *et al.*, 1990). The uptake was initiated by adding trace amounts of [^{14}C]TLC with 100 μM-TLC to cells incubated with or without PMA (0.1–100 nM) for 2 min. The results did not show any change in the bile acid uptake by the hepatocytes, whatever the concentration of PMA used. Fig. 4 shows an uptake of TLC with or without 100 nM-PMA, a concentration 30 times that which abolishes the TLC-mediated $[Ca^{2+}]_i$ rise (Fig. 2). The control [^{14}C]TLC influx, calculated from the slope of the regression line, was 2.49 ± 0.14 nmol/min per mg of cell protein ($n = 7$), a value similar to that published previously (Combettes *et al.*, 1990). The TLC influx measured in the presence of PMA was 2.80 ± 0.3 nmol/min per mg of protein ($n = 6$), i.e. not significantly different from that measured in untreated cells (Fig. 4). These results indicate that the inhibitory action of PMA on the TLC-induced Ca^{2+} response does not result from an inhibition of the transport mechanism responsible for concentrating TLC in hepatocytes.

Effect of PMA on TLC-mediated Ca^{2+} release from saponin-treated cells

A direct consequence of the results reported above is that PMA probably exerts a negative control over some step beyond the carrier-mediated accumulation of bile acids. Two possibilities may be proposed. First, PKC may inhibit the binding of bile acids to specific cytosolic proteins and/or their accumulation within vesicles responsible for intracellular transfer from the plasma membrane to the internal membranes (Hofmann, 1988; Stolz *et al.*, 1989; Erlinger, 1990), including that of the ER. Second, PKC may alter the site of action on the ER itself. An indirect action on the ER Ca^{2+} pump is less likely, because PMA does not affect the ability of the organelle to accumulate and to release Ca^{2+} in response to vasopressin in intact liver cells (Fig. 1). We examined these possibilities by studying the effect of PMA

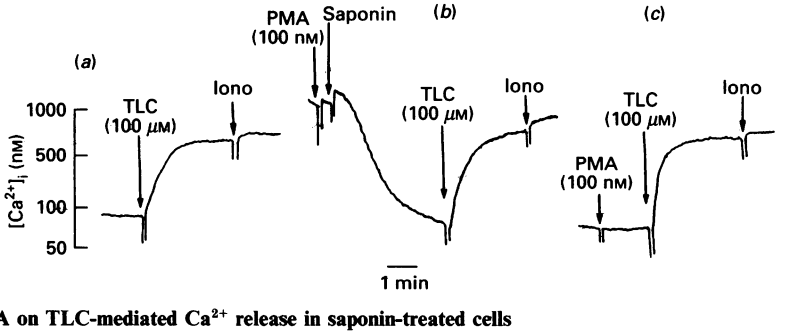

Fig. 5. Lack of effect of PMA on TLC-mediated Ca^{2+} release in saponin-treated cells

This figure shows a typical experiment performed in permeabilized hepatocytes. (*a*) The cells were incubated in the cytosol-like medium containing ATP (1.5 mM) and traces of Ca^{2+} (see the Materials and methods section). PMA (100 nM) was added either 2 min before saponin (*b*) or when the fluorescence signal had stabilized (*c*), indicating that Ca^{2+} uptake had reached equilibrium. Addition of TLC in each case raised the Ca^{2+} concentration in the medium from about 100 nM to 500 nM and released more than 80–90% of the Ca^{2+} mobilized by the Ca^{2+} ionophore ionomycin (Iono; 5 μM).

in cells permeabilized by saponin, (1) because TLC releases Ca^{2+} from the ER in this system (Combettes *et al.*, 1989), and (2) because the PMA-induced phosphorylation of endogenous proteins was maintained in permeabilized rat liver cells. Indeed, preliminary results showed a phosphorylation by PKC of at least two major endogenous proteins (120 and 210 kDa) in intact cells. In permeabilized cells pretreated with PMA, a similar phosphorylation of the same proteins was observed. However, when added after permeabilization, PMA failed to induce phosphorylation (results not shown). Cells were incubated in the cytosol-like medium containing ATP and no added Ca^{2+}. Upon addition of saponin (50 μg/ml), cells accumulated Ca^{2+} rapidly and decreased the contaminant Ca^{2+} from 3 μM to about 100 nM (Fig. 5). Under these conditions, the predominant compartment concentrating Ca^{2+} is the ER (Burgess *et al.*, 1984; Joseph *et al.*, 1984). As reported in previous studies (Combettes *et al.*, 1988*b*, 1989), the addition of TLC (100 μM) caused a rapid release of more than 80–90 % of the Ca^{2+} mobilized by the Ca^{2+} ionophore ionomycin (5 μM) (Fig. 5*a*). PMA, at maximal concentration (100 nM), added before or after cell permeabilization, did not affect the ability of TLC (100 μM) to cause Ca^{2+} to be released from permeabilized cells (Figs. 5*b* and 5*c*). PMA had no effect on the capacity of cells to concentrate Ca^{2+} in the ER, as final $[Ca^{2+}]_i$ values found in the medium were equal in controls and in PMA-treated cells, respectively 157 ± 27 nM and 170 ± 29 nM ($n = 4$; see also Fig. 5). Moreover, the amount of Ca^{2+} released by ionomycin was not altered by either of the experimental conditions used for PMA treatments (Fig. 5). Thus TLC-mediated Ca^{2+} release appears to be unaffected by PMA in permeabilized liver cells, presumably because the permeabilization methods dilute binding proteins and may alter the transfer of TLC from the plasma membrane to the internal membranes.

DISCUSSION

In the present work, we have shown that the increase in $[Ca^{2+}]_i$ effected by the monohydroxy bile salt TLC was inhibited by activation of PKC. That the action of phorbol esters is mediated by PKC-dependent phosphorylation is supported by the following evidence. First, the dose–response curve for PMA and the structure–activity relationships of different phorbol esters on the TLC-mediated $[Ca^{2+}]_i$ rise accord with the ability of these molecules to active PKC (Castagna *et al.*, 1982). Second, the effect of TPA was mimicked by the natural PKC activator OAG. Third, the PKC inhibitors H7 and AMG-C_{16} (Hidaka *et al.*, 1984; Kramer *et al.*, 1989) partly prevented the inhibitory action of PMA. Previous studies of hepatocytes and other types of cell have demonstrated feedback inhibition by phorbol esters of several receptor-mediated processes at the level of the plasma membrane, namely hormone binding, phospholipase C activation, inositol phosphate formation, and the subsequent mobilization of intracellular Ca^{2+} and activation of Ca^{2+}-dependent enzymes (Cooper *et al.*, 1985; Lynch *et al.*, 1985; Corvera *et al.*, 1986; for a review see Exton, 1988). However, phorbol esters in the liver have been reported to have differential effects on feedback inhibition of hormone-mediated responses. For example, PKC is involved in the down-regulation of α_1-adrenergic receptors, but not in the activation of the other $InsP_3$-dependent hormones, vasopressin and angiotensin II (Cooper *et al.*, 1985; Lynch *et al.*, 1985; Corvera *et al.*, 1986). The present results confirm that Ca^{2+} mobilization by the $InsP_3$-dependent peptide hormone vasopressin is resistant to PMA. We have shown previously that Ca^{2+} released by TLC is drawn from the same intracellular pool as that used by vasopressin in intact liver cells (Combettes *et al.*, 1988*b*) and that used by the messenger $InsP_3$ in saponin-treated cells, i.e. the ER (Combettes *et al.*, 1988*b*, 1989). However, despite a common intracellular target, the action of TLC differs in one respect from that induced by vasopressin. The effect of monohydroxy bile acids on cell Ca^{2+} is apparently independent of phospholipase C activation, polyphosphoinositide breakdown (Combettes *et al.*, 1988*b*) and the $InsP_3$ receptor (Combettes *et al.*, 1989; Capiod *et al.*, 1991). The effect of TLC is more likely to result from a primary and direct action on the permeability of the ER to Ca^{2+} (Combettes *et al.*, 1989), independent of an ionophore effect (Zimniak *et al.*, 1991). This is an important point, as it allows us to exclude the possibility that PKC feedback inhibits a small or local TLC-mediated production of $InsP_3$, as it does on $InsP_3$ synthesis by α_1-adrenergic receptors (Cooper *et al.*, 1985; Lynch *et al.*, 1985; Corvera *et al.*, 1986).

One of the key questions raised by the present work is how the phorbol-ester-mediated activation of PKC inhibits the Ca^{2+} rise induced by TLC in intact liver cells. Here, we have provided evidence that there is no direct link between activation of PKC and accumulation of the bile acid. The use of labelled TLC has allowed us to establish clearly that the binding of the bile acid to the carrier, its transfer through the plasma membrane and its accumulation by the cell are processes probably independent of PKC phosphorylation. The question was nevertheless relevant, since certain membrane transport systems are modulated by PKC (Nishizuka, 1986), and that the bile acid carrier(s) (Hofmann, 1988; Erlinger, 1990; Nathanson & Boyer, 1991) might have been, in common with these PKC-modulated carriers, under the close control of the enzyme activity. Also, the possibility that PKC abolishes the TLC-induced $[Ca^{2+}]_i$ rise by stimulating the Ca^{2+}-transport ATPase present in the plasma membrane of liver cells appears highly unlikely. In studies performed on other cells, PKC was reported to phosphorylate the plasma-membrane Ca^{2+} pump (Kuo *et al.*, 1991; Wang *et al.*, 1991) and to activate the outward Ca^{2+} transport in intact cells (Lagast *et al.*, 1984; Furukawa *et al.*, 1989). This transport, by removing Ca^{2+} from the cytosol, maintains the very low level of cytosolic $[Ca^{2+}]_i$ in resting cells. Thus PKC-mediated activation of the Ca^{2+} pump could reasonably be expected to decrease $[Ca^{2+}]_i$ (as shown elsewhere; Lagast *et al.*, 1984), therefore preventing the marked rise induced by the bile acid. However, the lack of any effect of PMA either on basal $[Ca^{2+}]_i$ or on the time course and maximal amplitude of the Ca^{2+} response initiated by the hormone vasopressin (Table 1) lends weight to the view that, under our experimental conditions, short-term activation of PKC does not stimulate the plasma-membrane Ca^{2+} pump in the liver.

PKC-mediated inhibition of the effect of the monohydroxy bile acid on $[Ca^{2+}]_i$ is highly reminiscent of PKC-mediated inhibition of bile secretion in the isolated perfused rat liver (Corasanti *et al.*, 1989). Both effects may result from a PKC-induced inhibition of a common step of the transfer of bile acids from the plasma membrane to other membranes. Intracellular translocation of bile acids is not a well-defined aspect of bile secretion. However, important data have pointed to the involvement of binding proteins and vesicular transport, in addition to free diffusion, in this transfer (Hofmann, 1988; Stolz *et al.*, 1989; Erlinger, 1990; Nathanson & Boyer, 1991). Since PKC appears to inhibit both normal bile secretion in perfused rat liver (Corasanti *et al.*, 1989) and the effect of the inhibitory bile acid TLC, the most plausible explanation for a common action of PKC is that it acts on the same intracellular step, i.e. on the translocation route common to trihydroxy and monohydroxy bile acids. The recovery of TLC-induced Ca^{2+} release in PMA-pretreated hepatocytes subsequently permeabilized by saponin is consistent with the view of a primary inhibitory action of PKC on the translocation mechanism. Permeabilization methods necessarily perturb this kind of transfer by diluting binding proteins

and probably also by disturbing vesicular transport. The investigation of the intracellular protein target of the action of PKC in the transfer of bile acids merits further study.

Through a great deal is known about the effects of the PKC system on hormonal responses in hepatocytes and in a wide variety of other cells (Nishizuka, 1986; Exton, 1988; Huang, 1989), little is known about the contribution of these effects to the transport and secretion of hepatic bile. Thus the inhibitory effect of phorbol esters and DAG might not be restricted to Ca^{2+}-mobilizing agents such as TLC, but rather may reflect a more general control of the metabolism of bile acids by PKC; therein might lie an explanation of the observed cholestasis generated by PKC activators (Corasanti *et al.*, 1989).

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