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Effect of the bile acid taurolithocholate on cell calcium in saponin-treated rat hepatocytes

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Neomycin was used to assess the involvement of Ins (1,4,5)P_3 in the Ca^{2+} release from the endoplasmic reticulum induced by the bile acid taurolithocholate. In saponin-permeabilized rat hepatocytes, neomycin via its ability to bind Ins (1,4,5)P_3 abolished the release of Ca^{2+} induced by added Ins (1,4,5)P_3. In contrast, it did not alter the Ca^{2+} release initiated by the bile acid. In intact cells, neomycin had no effect on the $[Ca^{2+}]_i$ rises promoted by taurolithocholate and vasopressin. It is suggested that the effect of taurolithocholate in liver is not mediated by Ins (1,4,5)P_3 but results from a primary action on endoplasmic reticulum.

Ca^{2+}; Bile acid; Endoplasmic reticulum; Neomycin; (Rat liver)

1. INTRODUCTION

It has been recently found that two natural bile acids, lithocholate (LC) and taurolithocholate (TLC), mobilize Ca^{2+} in rat liver [1,2]. In intact hepatocytes, these agents promote a fast and transient increase of the concentration of the cytosolic Ca^{2+}, $[Ca^{2+}]_i$, by mobilizing Ca^{2+} from the same pool as vasopressin [2]. The first action of this hormone in liver is to cause a rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and synthesis of inositol 1,4,5-trisphosphate [Ins (1,4,5)P_3] [3–7] that permeabilizes the endoplasmic reticulum (ER) to Ca^{2+} [8,9]. When applied to saponin-permeabilized hepatocytes, TLC and LC induce the release of Ca^{2+} from a non-mitochondrial, ATP-dependent pool that is sensitive to Ins (1,4,5)P_3 [2].

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Abbreviations: quin2, 2-[[2-bis(carboxymethyl)amino-5-methylphenoxyl]methyl]-6-methoxy-8-bis(carboxymethyl)-aminoquinoline; quin2/AM, quin2 tetraacetoxymethyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

Although TLC and LC mimick the action of vasopressin in intact cells and that of Ins (1,4,5)P_3 in saponin-treated cells, it does not alter the basal Ins (1,4,5)P_3 content of cells [2]. Because of this latter observation, we proposed that the bile acid-induced Ca^{2+} release was not mediated by PIP_2 breakdown [2]. However, we could not rule out the possibility that a small PIP_2 pool was being broken down by direct stimulation of phospholipase C by TLC and LC. This possibility is reinforced by the fact that in liver cells, Ins (1,4,5)P_3 is so rapidly metabolized and recycled to form PIP_2 (within a few tens of seconds, see [10–13]) that measurements of the level of the inositol phosphate may substantially underestimate the extent of its formation.

Here we have used the cationic aminoglycoside antibiotic neomycin as a probe to assess the involvement of Ins (1,4,5)P_3 in the TLC-mediated Ca^{2+} release in permeabilized hepatocytes. Neomycin, in addition to its ability to bind to polyphosphoinositides [14–19] has been shown to bind to molecules bearing polyphosphate groups, including Ins (1,4,5)P_3, thus inhibiting Ca^{2+} mobilization in permeabilized cells [20]. We show that neomycin abolishes the Ins (1,4,5)P_3-mediated

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Ca^{2+} release and has no effect on that promoted by TLC, demonstrating that the effect of the bile acid is not mediated by Ins (1,4,5)P_3, but results from a direct action on ER.

2. MATERIALS AND METHODS

2.1. *Materials*

Materials used for these studies were obtained from the following sources: neomycin sulfate from Sigma, quin2 and quin2/AM from Lancaster Synthesis, ionomycin from Calbiochem and Ins (1,4,5)P_3 from Amersham. All other chemicals were purchased from Sigma and were of the highest purity commercially available.

2.2. *Methods*

Rat hepatocytes were isolated and maintained (2×10^6 cells per ml) in an Eagle's medium containing 116 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.92 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 5.6 mM glucose as indicated [21]. The medium also contained 15 mg per ml gelatin and vitamins and amino acids. It was gassed with 95% O_2/5% CO_2 (pH 7.4) at 37°C.

The $[Ca^{2+}]_i$ in intact hepatocytes was measured by loading cells (2×10^6 cells per ml) with 50 μM quin2/AM for 150 s. Cells were washed and transferred into the cuvette of the spectrofluorimeter (0.4×10^6 cells per ml) at 37°C, under continuous magnetic agitation and gassing with 95% O_2/5% CO_2 and $[Ca^{2+}]_i$ was calibrated and corrected for autofluorescence as in [21].

Rat hepatocytes (3×10^6 cells per ml) were permeabilized as reported by Burgess et al. [22]. The cells were incubated in an 'internal medium' containing 20 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 25 mM Hepes (pH 7.15 with KOH) at 37°C. The medium also contained 5 μM of the mitochondrial uncoupler CCCP, 1.5 mM ATP, 5 mM creatine phosphate and 5 U creatine phosphokinase per ml and 10 μM quin2. The concentration of contaminant Ca^{2+} in this control medium as measured by atomic absorption spectroscopy was 3.26 ± 0.25 μM ($n = 7$). In the presence of 1 mM neomycin, the contaminant Ca^{2+} was increased to 7.48 ± 0.39 μM ($n = 5$) indicating that the antibiotic contains traces of Ca^{2+}. So, the concentration of quin2 was increased to 20 μM in this medium. The concentration of free Ca^{2+} and the amount of Ca^{2+} released by the cells were calculated as in [21]. Determination of protein content was carried out by the Lowry method using serum albumin as standard protein.

3. RESULTS

3.1. *Effect of neomycin on intact cells*

The effects of TLC and vasopressin on $[Ca^{2+}]_i$, at concentrations of 100 μM and 10 nM, respectively, which promote maximal Ca^{2+} movements in liver [2–8], are shown in fig.1. In the presence of EGTA (1.24 mM) which abolishes Ca^{2+} influx [21,23], the bile acid and the hormone initiated transient rises of $[Ca^{2+}]_i$. Examination of the

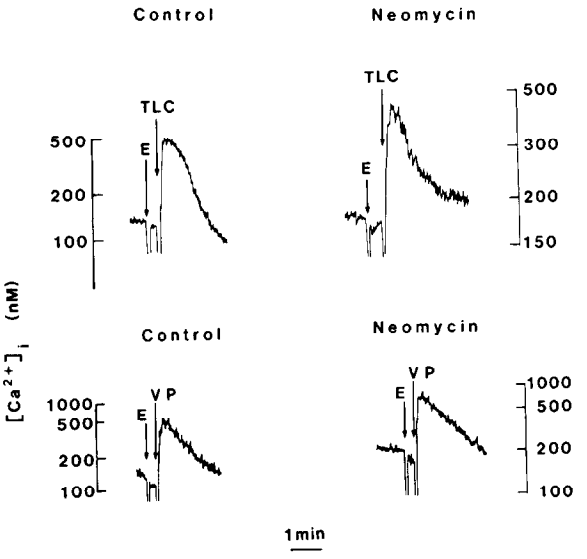

Fig.1. Lack of effect of neomycin on vasopressin- and TLC-mediated $[Ca^{2+}]_i$ rises in intact cells loaded with quin2. Hepatocytes were incubated in an Eagle's medium containing 1.2 mM Ca^{2+}, then EGTA (E, 1.24 mM) which reduced the concentration of external Ca^{2+} to less than 6 μM [21]. Maximal concentrations of TLC (TLC, 100 μM) and vasopressin (VP, 10 nM) were added 15 s after EGTA. Addition of the same volumes of the respective solvents (4 μl DMSO for TLC and deionized water for vasopressin and neomycin) had no effect on the $[Ca^{2+}]_i$. Neomycin (1 mM) was added 2 min before EGTA.

kinetics of the Ca^{2+} response indicated that the peak was maximal at 20 s with TLC and less than 6 s with vasopressin. The amplitudes of the $[Ca^{2+}]_i$ rises were not different: TLC increased $[Ca^{2+}]_i$ from 170 ± 9 to 609 ± 44 nM and vasopressin to 560 ± 63 nM ($n = 12$). The $[Ca^{2+}]_i$ then slowly returned to basal level within 2–3 min. We have shown that these $[Ca^{2+}]_i$ rises result from the permeabilization of the same internal pool, ER [2]. The decreasing phase represents the net extrusion of Ca^{2+} from the cells [2]. The action of TLC was dose-dependent with a half-maximal effect observed at 20 μM. The possibility of artifactual Ca^{2+} contamination by TLC was assessed by atomic absorption spectroscopy. No contamination was detected by this procedure: the Ca^{2+} concentration of Eagle's medium was not affected by TLC (100 μM) and no Ca^{2+} was detected when the agent was added to deionized water.

Fig.1 shows the effect of neomycin (1 mM) added 2 min before the agents on $[Ca^{2+}]_i$ of intact

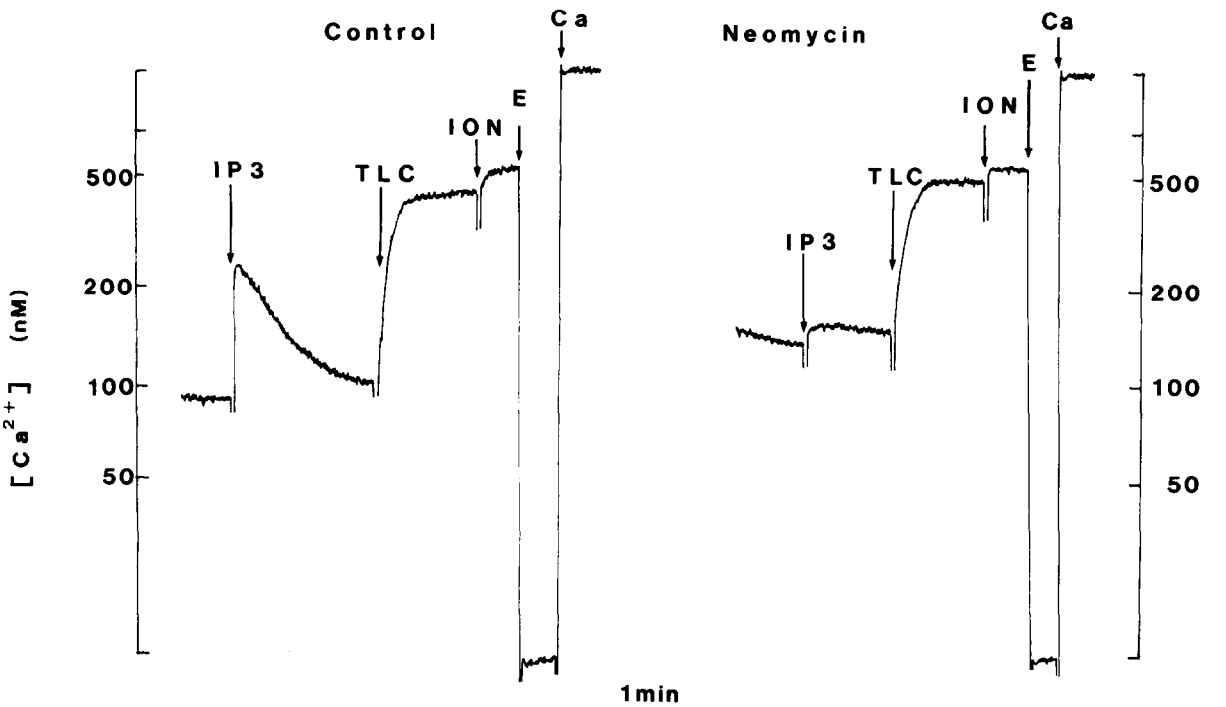

Fig.2. Neomycin reduces Ins (1,4,5)P_3-mediated Ca^{2+} release and has no effect on that initiated by TLC in saponin-treated hepatocytes. The cells were incubated in the internal medium containing ATP (1.5 mM) and regenerating system, CCCP (5 μM) and quin2. On saponin addition, the cells accumulated the contaminant Ca^{2+} and Ins (1,4,5)P_3 (IP3, 1 μM), TLC (100 μM) then ionomycin (ION, 5 μM) were added when ER had lowered the medium free $[Ca^{2+}]$ to about 100 nM. Quin2 fluorescence was calibrated by successive additions of EGTA (300 μM) then Ca^{2+} (2.5 mM). Neomycin (1 mM) was added 2 min before Ins (1,4,5)P_3 and TLC.

hepatocytes. As expected from a large non-permeant molecule, neomycin had no effect on basal $[Ca^{2+}]_i$ and on the response induced by TLC or vasopressin. This is in agreement with other studies showing that the cell membrane has to be permeabilized to allow neomycin access to the inner leaflet of the plasma membrane where polyphosphoinositides are located [15–17,20, 24–26].

3.2. *Effect of neomycin in saponin-treated cells*

To test whether the effects of TLC were indirectly mediated by Ins (1,4,5)P_3, neomycin was used in hepatocytes permeabilized by saponin [22]. Intact cells were incubated in the internal medium with high $[K^+]$, low $[Na^+]$, ATP and regenerating system, the mitochondrial uncoupler CCCP and no added Ca^{2+}. Under these conditions, the predominant compartment concentrating Ca^{2+} is ER [8,9,22]. On saponin addition (50 μg/ml), cells accumulated Ca^{2+} rapidly and decreased the contaminant Ca^{2+} from 3 μM (see section 2) to about 100 nM (fig.2). The addition of Ins (1,4,5)P_3 (1 μM) and TLC (100 μM) caused a rapid release of Ca^{2+} whereas no increase in the fluorescence emission was detected when both agents were added to a cell-free internal medium. In contrast to TLC, the messenger-mediated Ca^{2+} release (fig.2) was transient presumably because of rapid degradation of Ins (1,4,5)P_3 by cell phosphatases [10–13]. The dose-response curves for the action of TLC and Ins (1,4,5)P_3 are shown in fig.3A,B. The half-maximal effects were observed with 300 nM Ins (1,4,5)P_3 and 20 μM TLC. Maximal concentrations of TLC and Ins (1,4,5)P_3 released 90% of the Ca^{2+} mobilized by the Ca^{2+} ionophore ionomycin (5 μM). The total Ca^{2+} mobilized by TLC and by the messenger was about 0.5 nmol/mg cell protein, which approximates the amounts of Ca^{2+} mobilized by vasopressin in intact hepatocytes [4,6].

When added to the internal medium lacking

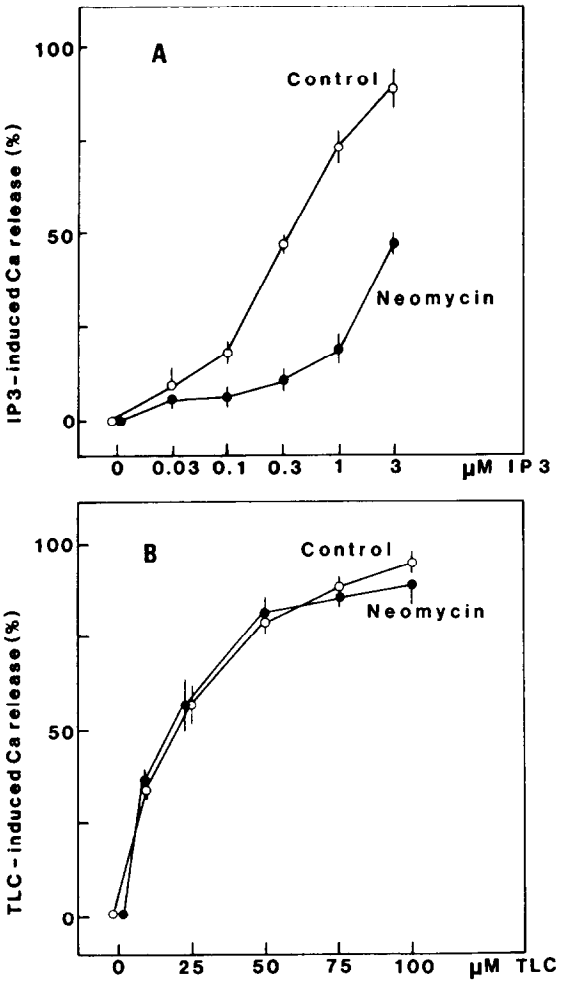

Fig.3. Effect of neomycin on the Ca^{2+} release at different concentrations of (A) Ins $(1,4,5)P_3$ and (B) TLC in permeabilized hepatocytes. The Ca^{2+} mobilized from the cells was expressed as a percentage of the Ca^{2+} released by ionomycin (5 μM) and was calculated as indicated in section 2.

cells, neomycin (1 mM) did not alter the optical properties of the Ca^{2+} indicator quin2, maximal binding or affinity of the molecule for Ca^{2+}. In the presence of permeabilized hepatocytes, it had no effect on the ability of cells to concentrate Ca^{2+} in ER, as illustrated by the fact that the final $[Ca^{2+}]$ values found in the medium were equal in controls and in neomycin-treated cells (respectively 176 ± 13 nM, $n = 58$ and 158 ± 7.1 nM, $n = 43$). Fig.2B shows the effect of neomycin on the Ca^{2+} release initiated by Ins $(1,4,5)P_3$ and TLC. In agreement with other studies [20,26], a concentration of 1 mM neomycin markedly impaired the ability of Ins $(1,4,5)P_3$ (1 μM) to release Ca^{2+} from permeabilized cells. In contrast, the antibiotic did not alter the TLC-mediated Ca^{2+} release. The results of experiments performed at different concentrations of Ins $(1,4,5)P_3$ and TLC are shown in fig.3. The effect of 1 μM Ins $(1,4,5)P_3$ that released 75% of the Ca^{2+} accumulated by the control cells was reduced to 19% in the presence of the antibiotic. Consistent with the observation reported by Prentki et al. [20] on Ins $(1,4,5)P_3$ binding by neomycin, the drug shifted the dose-response curve of Ins $(1,4,5)P_3$ action. In other terms, neomycin markedly inhibited the effects of low concentrations of Ins $(1,4,5)P_3$ and the magnitude of the inhibition progressively diminished as the Ins $(1,4,5)P_3$ concentration was increased. From the data shown in fig.3, it may be estimated that about 10-times higher concentrations of Ins $(1,4,5)P_3$ were required for recovering the same effect of the messenger. This effectiveness of neomycin in reducing the effect of Ins $(1,4,5)P_3$ and its lack of effect on the TLC-mediated Ca^{2+} release support the view that bile acid mobilizes Ca^{2+} via a direct action on ER in liver cells.

4. DISCUSSION

Neomycin is a polycationic molecule which binds to polyphosphoinositides [14], whose degradation by phospholipase C leads to formation of Ins $(1,4,5)P_3$ and diacylglycerol [3–7]. So, it has been largely used to assess the involvement of PIP_2 breakdown in the stimulation-response coupling of a variety of cell functions [15–19,25,26]. In a recent study, Prentki et al. [20] showed that in addition to polyphosphoinositides, neomycin is able to bind to other polyanionic molecules, particularly Ins $(1,4,5)P_3$ which accounts for the inhibition of the Ca^{2+} release induced by added Ins $(1,4,5)P_3$ in permeabilized cells. In the present work, we have used this property of the molecule to bind to Ins $(1,4,5)P_3$ to assess the involvement of the intracellular messenger in the TLC-mediated Ca^{2+} release in liver. Neomycin does not affect the ability of ER to pump actively Ca^{2+} in saponin-treated hepatocytes. In agreement with Prentki et al. [20], it markedly inhibits the Ca^{2+} release induced by Ins $(1,4,5)P_3$ in rat liver cells. Since the antibiotic did

not affect the releasing action of TLC, our results demonstrate that the effect of TLC is not mediated by Ins (1,4,5)P_3. The question is of importance since TLC mimicks the action of the PIP_2-dependent hormone vasopressin in intact hepatocytes and the effect of Ins (1,4,5)P_3 in permeabilized cells [1,2]. Our results rule out the possibility that the bile acid could have promoted a local increase of Ins (1,4,5)P_3 in the vicinity of messenger receptors [28], too small to be easily detected by chromatography but large enough to cause Ca^{2+} release from ER.

The mechanism by which TLC controls the Ca^{2+} permeability of ER is not yet known [2]. An ionophore-like effect on Ca^{2+} permeability appears unlikely, since TLC has been shown to permeabilize selectively ER without altering ion permeabilities of plasma and mitochondrial membranes [2]. An ionophore-like effect is not likely to be specific for a particular membrane. It is possible that TLC might bind to specific receptors and permeabilize the membrane of liver ER in a way similar (but independent) to Ins (1,4,5)P_3 [8,9,29].

The natural bile acids are rapidly accumulated by hepatocytes from plasma, then actively secreted at the canalicular pole of cell [31,32]. This net transport of bile acids is the driving force responsible for native bile formation. The finding that TLC permeabilizes ER strongly supports a role for Ca^{2+} in this process. The monohydroxylated bile acids such as TLC and LC are known to reduce dramatically bile secretion when injected into rats or hamsters [32,33]. Both mobilize Ca^{2+} from ER in intact cells and in saponin-treated hepatocytes ([2] and present results for the action of TLC). Since bile secretion is believed to be dependent on Ca^{2+} (see [33] for references), it is conceivable that ER permeabilization to Ca^{2+} could be at the origin of the inhibition of bile secretion. This internal compartment plays a major role in the control of cell Ca^{2+} [6,7]. A sustained permeabilization of ER by TLC should alter Ca^{2+}-dependent functions, including bile acid secretion and cell hormone responses.

In conclusion, neomycin, via its ability to bind inositol polyphosphate groups, discriminates the Ca^{2+} releases initiated by the bile acids and Ins (1,4,5)P_3. This suggests that the bile acid increases $[Ca^{2+}]_i$ and stimulates cell Ca^{2+} efflux in intact hepatocytes [2] by directly permeabilizing ER.

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