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Silibinin prevents cholestasis-associated retrieval of the bile salt export pump, Bsep, in isolated rat hepatocyte couplets: Possible involvement of cAMP☆

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Abstract

Estradiol-17ß-D-glucuronide ($E_2$17G) and taurolithocholate (TLC) induce acute cholestasis-associated with retrieval of the bile salt export pump (Bsep), which parallels with alteration in transport activity. cAMP stimulates the apically directed vesicular trafficking of transporters, partially preventing these alterations. The hepatoprotector, silymarin, which inhibits cAMP-phosphodiesterase, prevents the cholestasis induced in vivo by both estrogens and TLC.

We aimed to assess the ability of silibinin (Sil), the silymarin active component, to prevent the retrieval of Bsep induced by TLC and $E_2$17G, and the associated alteration in its transport function. The possible involvement of cAMP as a second messenger and the intracellular signalling pathways implicated were also evaluated. Functional studies were performed analysing the proportion of isolated rat hepatocyte couplets (IRHC) accumulating the fluorescent bile salt analogue, cholyl-lysylfluorescein (CLF), into their sealed canalicular vacuoles. Cellular localisation of Bsep was assessed by immunofluorescent staining. Intracellular levels of cAMP were measured by ELISA. Sil (2.5 μM) elevated by 40 ± 3% intracellular cAMP, and mimicked the ability of dibutyryl-cAMP (10 μM) to prevent internalisation of Bsep and the TLC (2.5 μM)- and $E_2$17G (50 μM)-induced impairment in the capacity of IRHC to accumulate CLF apically. Preventive effects of Sil and dibutyryl-cAMP were not abolished by the specific protein kinase A inhibitors, KT5720 and H89. Contrarily, the intracellular Ca^{2+} chelator, BAPTA/AM, significantly blocked the protective effect of both compounds.

We conclude that Sil prevented TLC- and $E_2$17G-induced bile salt secretory failure, at least in part, by avoiding redistribution of Bsep, by a mechanism probably involving cAMP-induced cytosolic Ca^{2+} elevations.

Keywords: Estradiol-17ß-D-glucuronide; Taurolithocholate; Bsep; Ca^{2+}- and PKA-dependent signalling pathways; Dibutyryl-cAMP; Isolated rat hepatocyte couplets

1. Introduction

Bile formation is an osmotic process driven by the active secretion of solutes into the bile canaliculus across the hepatocyte apical membrane, followed by passive inflow of water [1]. BS are quantitatively the major constituents of bile, and the main determinants of its volume. Active, concentrative transport of tauro- and glycoconjugated BS through the canalicular membrane is principally mediated by Bsep (Abcb11), a member of the ATP-binding cassette superfamily of membrane transporters [2].

The cholestatic BS, TLC [3], and the endogenous estrogen metabolite, $E_2$17G [4], induce an acute, reversible

Abbreviations: BS, bile salts; Bsep, bile salt export pump; TLC, taurolithocholate; $E_2$17G, estradiol-17ß-D-glucuronide; DBcAMP, N6,2′-*o*-dibutyryladenosine 3′,5′-cyclic monophosphate, dibutyryl-cAMP; IRHC, isolated rat hepatocyte couplets; Sil, silibinin; L-15, Leibovitz-15 culture medium; CLF, cholyl-lysylfluorescein; cVA, canalicular vacuolar accumulation; PKA, protein kinase A; Ntcp, sodium taurocholate cotransporting polypeptide

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cholestasis in the rat. These cholestatic agents affect both the BS-dependent and the BS-independent fractions of the bile flow. The mechanisms underlying this harmful effect are largely unknown. We have recently shown that both $E_2$17G and TLC induce rapid endocytic internalisation of Bsep [5,6], thus accounting, at least in part, for the impairment in BS secretion induced by these cholestatic agents. Retrieval of Mrp2, thus impairing the Mrp2-dependent transport of glutathione to the canaliculus [7], was also reported to occur in $E_2$17G- [8] and TLC-induced cholestasis [9]; glutathione is thought to contribute to the bile salt independent bile flow [10]. Furthermore, we found that pre-treatment with dibutyryl-cAMP (DBcAMP), a permeable analogue of cAMP which stimulates the trafficking of intracellular vesicles containing membrane transporters into the canalicular pole [11–13], partially prevented both $E_2$17G- and TLC-induced alteration in localisation and function of Bsep [5,6] and Mrp2 [8], both in vivo and in IRHC.

Silymarin is a purified extract from the milk thistle *Silybum marianum* (L.) Gaertn, which is composed of a mixture of four isomeric flavonolignans, namely: Sil, isosilibinin, silidianin and silichristin [14]. Sil, which constitutes 60–70% of the silymarin mixture, has been identified as the major active component [15]. Extracts of milk thistle have been empirically used as hepatoprotective agents from ancient times, and found to have beneficial effects in several hepatic disorders [15,16]. In recent studies, we have shown that silymarin protects against the cholestatic manifestations induced by estrogens [17] and TLC [18]. These beneficial effects include improvement of canalicular transport of BS and other cholephilic organic anions [17,18]. The mechanisms involved in the prevention of the alteration of transport at a canalicular level are at present unknown, but could involve prevention of canalicular transporter's retrieval induced by these cholestatic agents. A beneficial effect of silymarin by preventing canalicular transporter internalisation is likely, since the components of silymarin were found to be potent inhibitors of the enzyme involved in cAMP degradation, cAMP phosphodiesterase [19]; the resultant increase in intracellular levels of cAMP could be responsible for the prevention of canalicular transporter retrieval. The aim of this work was, therefore, to assess the ability of Sil, the active component of silymarin, to prevent the retrieval of Bsep induced by $E_2$17G and TLC, and the associated alteration in its secretory function in IRHC. Bearing in mind that both cAMP and silymarin share anticholestatic properties, another major aim of this study was to examine the possible involvement of cAMP as a mediator of Sil effects, by establishing analogies between both hepatoprotectors in the prevention of the impairment in Bsep localisation/function induced by $E_2$17G and TLC, as well as in the signalling pathways involved in their anticholestatic effects.

2. Materials and methods

2.1. Chemicals

Sil, TLC (sodium salt), $E_2$17G, L-15 culture medium, DMSO and DBcAMP were from Sigma Chemical Co. Cholyl-lysylfluorescein (CLF) was kindly provided by Dr. Charles O. Mills. Collagenase type A from *Clostridium histolyticum* was from Gibco. KT5720, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-89), and 1,2-bis-(*o*-aminophenoxy)-ethene-*N*,*N*,*N*′,*N*′-tetraacetate tetra-(acetomethyl)ester (BAPTA/AM) were obtained from Alexis Co. All other chemicals were of the highest grade available.

2.2. Animals

Adult, male Wistar rats weighing 300–350 g were used throughout. Animals were maintained on a standard diet and water ad libitum, under a constant 12 h-light/12 h-dark cycle. Protocols were conducted according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the NIH (publication 25-28, revised 1996), and regulations stated by the Faculty of Biochemical and Pharmaceutical Sciences of the National University of Rosario (Argentina).

2.3. Experiments in isolated hepatocytes

2.3.1. Hepatocyte isolation

Isolated hepatocytes were obtained by collagenase perfusion of rat liver, as previously described [20]. Cell viability, evaluated by the trypan blue exclusion test [21], was >90% in all cellular preparations.

2.3.2. Assessment of intracellular cAMP levels

Isolated hepatocytes (1×10^6 cells/ml, 2 ml per dish) were incubated with Sil (2.5 μM) or DMSO (control group) for 30 min in a buffer medium containing NaCl 139 mM, Na_2HPO_4 0.33 mM, KCl 5.4 mM, KH_2PO 40.44 mM, Hepes 24.2 mM, glucose 5.6 mM, $CaCl_2$ 2.5 mM and $MgSO_4$ 1.1 mM, at 37 °C under oxygen atmosphere. After incubation, intracellular cAMP levels were measured by ELISA (cAMP Biotrak Enzymeimmunoassay System, Amersham Biosciences), following manufacturer's instructions.

2.3.3. Assessment of CLF uptake

This study was performed to evaluate the influence of the different treatments on hepatocellular BS uptake. Cells (2×10^6 units/ml) were preincubated with DBcAMP (10 μM) or Sil (2.5 μM) for 30 min. Then, TLC (2.5 μM) or $E_2$17G (50 μM) were added, and the cells incubated for another 20 min. The uptake assays consisted of 0.3 ml hepatocyte suspension in a 1.5 ml

microcentrifuge tube kept at 37 °C, to which CLF (2 μM, final concentration) was added at time = 0, and incubated for the appropriate periods of time (0 or 45 s routinely, 0–300 s in time course studies); time = 0 uptake experiments were performed to discount fluorescence due to unspecific binding of CLF.

The tube was then centrifuged at 13000 rpm for 5 s, the start of centrifugation being considered the end of the incubation period. The pellet was rapidly rinsed twice with 1 ml of PBS, dissolved in 0.3 ml 1% Triton X-100 in PBS, and made up to 3 ml with PBS. Fluorescence of this solution was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Shimadzu RF-5301 PC).

2.4. Experiments in IRHC

2.4.1. Hepatocyte couplet isolation, enrichment and culture

IRHC were obtained from rat liver, according to the two-step collagenase perfusion procedure described by Wilton et al. [22], adapted from Gautam et al. [23], and further enriched by centrifugal elutriation [22]. The final preparation, containing 70–80% of IRHC with viability >95%, as assessed by the trypan blue exclusion test, was plated in L-15 medium [24] containing penicillin/streptomycin onto 35-mm plastic culture dishes (2 ml/dish), at a density of 0.5×10^5 hepatocyte units (singles plus multiples) per ml, and incubated at 37 °C for 4.5–5 h prior to experimentation to allow restoration of couplet polarity.

2.4.2. Assessment of BS secretory function

BS secretory function was evaluated by carrying out the cVA of CLF test, which assesses the percentage of couplets present in the field displaying sufficient CLF in their canalicular vacuoles to make it visible under inverted fluorescent microscopy, from a total counting of at least 50 couplets per preparation [25,26]; this test was carried out after 15 min of CLF exposure, when a maximum secretory stationary state has been reached [25]. Since canalicular secretion rather than uptake is the rate-limiting step in the overall hepatocellular transport of BS [27], this parameter more likely reflects changes in the former, as the period of time allowed for CLF to accumulate apically (i.e., 15 min) is long enough to compensate for any change in the uptake process.

In some experiments, intracellular CLF fluorescence was quantified. Microphotographs from randomly chosen vision fields were digitalized, and CLF-associated green fluorescence in the cellular body quantified by densitometry, using an image analysis program (Openlab, Improvision). Intracellular fluorescence intensity was determined as the total fluorescence in the cytoplasm of the couplets (∼ 40) divided by the cytoplasmatic area.

2.4.3. Assessment of Bsep localisation

For Bsep immunofluorescent staining, IRHC were fixed with 4% paraformaldehyde in PBS, and incubated with a polyclonal anti-Bsep rabbit antibody (1:250, 2 h) (Kamiya Biomedical Company), followed by incubation with FITC-labelled goat anti-rabbit IgG (1:100, 40 min) (Zymed). Cells were then mounted and examined by fluorescence microscopy (Zeiss Axiovert 350TV, equipped with plan-neofluar lenses).

Monochrome images (20–30 per group) were taken in 1-μm steps, and captured on a CCD video camera (Hamamatsu Photonic Sys. Corp.). Out-of-focus flair was removed using a deconvolution program (Micro-Tome Mac). Quantification of Bsep-associated fluorescence in the canalicular area, expressed as the percentage of total (canalicular membrane plus cell body) fluorescence intensity, was achieved by using Openlab software (Improvision). The canalicular space was identified on Bsep-labelled IRHC by superposing each fluorescent image with its respective phase contrast image [5].

2.4.4. Treatments

In studies aimed to assess the effect of the cholestatic agents studied on cVA of CLF, couplets were exposed to DMSO (control group), TLC (2.5 μM, in 2 μl of DMSO) [6] or $E_2$17G (50 μM, in 2 μL of DMSO) [5,28], for 20 min. Then, the cholestatic compounds were removed by washing twice with L-15, and cells were exposed to CLF (2 μM) for 15 min. Next, CLF was removed, and cVA of CLF was assessed by using an inverted fluorescence microscope (Olympus IMT2-RFL, Olympus Optical Ltd.).

To assess the ability of Sil and DBcAMP to prevent the impairment in cVA of CLF induced by TLC and $E_2$17G, IRHC were preincubated with DBcAMP (10 μM) [13,29] or Sil (2.5 μM) [17] for 30 min. Then, TLC (2.5 μM) or $E_2$17G (50 μM) were added, and cells were incubated for another 20 min; cVA of CLF test was then performed as described above. Sil was removed from the incubation medium before adding the cholestatic agents, to avoid putative competition for uptake between these compounds.

DBcAMP induces both PKA activation and elevations of intracellular Ca^{2+} [30,31]. In studies aimed to assess whether any of these signalling molecules are involved in the anticholestatic properties of Sil and DBcAMP, IRHC were preincubated for 15 min with either of the PKA inhibitors, KT5720 (5 μM) [13,29] or H-89 (0.1 μM) [13], or with the intracellular Ca^{2+} chelator, BAPTA/AM (20 μM). DMSO (10 μl) was added in the control group. Then, the hepatoprotective compounds and/or the cholestatic agents were added as described above.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. One way ANOVA, followed by Newman-Keuls test, was performed for multiple comparison. Statistical analysis of cAMP levels was performed by using the paired Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of Sil on hepatocellular cAMP levels

The capability of Sil to increase cAMP levels was assessed in isolated rat hepatocytes. Determinations performed by triplicate from experiments carried out in three independent hepatocyte preparations showed that Sil increased the intracellular levels of cAMP by $40 \pm 3\%$ ($p < 0.05$) over the control value.

3.2. Effect of the different treatments on CLF uptake by isolated hepatocytes

Functional status of the main sinusoidal BS uptake system of hepatocytes, Ntcp, was evaluated in isolated hepatocytes. Time-course studies showed that CLF uptake was linear until 100 s (data not shown); thus, 45 s were chosen for routine assays.

As shown in Table 1, $E_2$17G significantly inhibited CLF uptake by isolated hepatocytes, Sil and DBcAMP being unable to prevent this alteration. On the contrary, TLC-treated hepatocytes showed only a non-significant trend towards a diminished uptake, probably associated to competition between CLF and TLC for Ntcp; Sil and DBcAMP did not modify this response.

3.3. Effect of Sil and DBcAMP on TLC- and $E_2$17G-induced decrease in cVA of CLF

Functional status of the canalicular BS export pump, Bsep, was evaluated in IRHC by using the cVA of CLF test. As shown in Table 2, $68 \pm 5\%$ of couplets exhibited CLF-associated fluorescence into their canalicular vacuoles under control conditions. Both TLC and $E_2$17G significantly decreased this value by 52 and 57%, respectively, and DBcAMP pre-treatment virtually normalised this parameter. Sil pre-treatment fully mimicked DBcAMP protective effect in both cholestatic conditions (Table 2). Neither Sil nor DBcAMP had any effect per se on cVA of CLF (data not shown).

Table 2
Effect of Sil and DBcAMP on the impairment of canalicular accumulation of bile salts induced by $E_2$17G and TLC[a]

cVA of CLF (%)[b]	
Control	68 ± 5
TLC	$33 \pm 4^{*}$
TLC + Sil	$63 \pm 4^{**}$
TLC + DBcAMP	$65 \pm 3^{**}$
$E_2$17G	$29 \pm 1^{*}$
$E_2$17G + Sil	$65 \pm 2^{***}$
$E_2$17G + DBcAMP	$61 \pm 4^{***}$

DBcAMP, N^6,2′-*o*-dibutyryladenosine 3′,5′-cyclic monophosphate, dibutyryl-cAMP; Sil, silibinin; $E_2$17G, estradiol-17ß-D-glucuronide; TLC, taurolithocholate; CLF, cholyl-lysylfluorescein; cVA, canalicular vacuolar accumulation.

[a] Values are expressed as mean ± S.E.M. for six independent cellular preparations per group.

[b] Hepatocyte couplets in suspension were pretreated with DBcAMP or Sil for 30 min at 37 °C before exposing them to either TLC or $E_2$17G for another 20 min. Controls receiving only the vehicle (DMSO) were also run. cVA of CLF test was performed as described in Section 2.

[*] Different from control group ($p < 0.01$).

[**] Different from TLC ($p < 0.01$).

[***] Different from $E_2$17G ($p < 0.01$).

3.4. Effect of Sil and DBcAMP on TLC- and $E_2$17G-induced changes in CLF intracellular content

As can be seen in Table 1, both TLC and $E_2$17G increased cytosolic CLF fluorescent intensity in IRHC by 72 and 22%, respectively. This strongly suggests that canalicular secretory failure is due to impaired transport across the canalicular membrane rather than to a decreased uptake. Furthermore, CLF intracellular levels were restored to normality following either DBcAMP or silibinin administration, clearly suggesting that the improved canalicular secretion was due to an improved canalicular transport of CLF.

3.5. Effect of Sil and DBcAMP on TLC- and $E_2$17G-induced internalisation of Bsep

Bsep immunofluorescent staining in IRHC is depicted in Fig. 1. In control conditions, Bsep was mainly confined to the canalicular membrane. Neither Sil nor DBcAMP

Table 1
Effect of the different treatments on CLF uptake by isolated hepatocytes and CLF intracellular content in IRHC

	Control	TLC	TLC + Sil	TLC + DBcAMP	$E_2$17G	$E_2$17G + Sil	$E_2$17G + DBcAMP
CLF uptake (percent of control)[a]	100	88 ± 7	86 ± 6	81 ± 8	$56 \pm 1^{*}$	$56 \pm 2^{*}$	$59 \pm 2^{*}$
CLF intracellular content (fluorescence intensity)[b]	36 ± 2	$62 \pm 5^{*}$	37 ± 3	37 ± 2	$44 \pm 2^{*}$	36 ± 1	35 ± 2

DBcAMP, N^6,2′-*o*-dibutyryladenosine 3′,5′-cyclic monophosphate, dibutyryl-cAMP; Sil, silibinin; $E_2$17G, estradiol-17ß-D-glucuronide; TLC, taurolithocholate; CLF, cholyl-lysylfluorescein.

[a] Isolated hepatocytes in suspension were pretreated with DBcAMP or Sil for 30 min at 37 °C before exposing them to either TLC or $E_2$17G for another 20 min. Controls receiving only the vehicle (DMSO) were also run. Uptake of CLF was assessed as described in Section 2. Values are expressed as mean ± S.E.M. for three independent cellular preparations per group.

[b] IRHC in culture were pretreated with DBcAMP or Sil for 30 min at 37 °C before exposing cells to either TLC or $E_2$17G for another 20 min, followed by CLF exposure for a further 15-min period. Controls receiving only the vehicle (DMSO) were also run. CLF intracellular fluorescence intensity (arbitrary units) was assessed as described in Section 2. Values are expressed as mean ± S.E.M. for approximately 40 couplets, randomly selected from microphotographs taken from three independent cellular preparations per group.

[*] Different from control group ($p < 0.05$).

affected this normal localisation. TLC and $E_2$17G induced a marked Bsep relocalisation into vesicles localised in the entire cell body, a finding compatible with endocytic internalisation of this transporter; this relocalisation was extensively prevented by DBcAMP. As occurred with Bsep function, Sil was also instrumental in preventing cholestasis-associated changes in Bsep localisation. Contrarily, no change in the normal IRHC topography (as visualised by phase contrast microscopy) was induced by any of the treatments (Fig. 1, insets).

Quantification of the retrieval of Bsep is shown in Fig. 2. The proportion of fluorescence intensity present in canalicular membrane was diminished by 58% by TLC, and by 45% by $E_2$17G. DBcAMP pre-treatment virtually normalised Bsep localisation in both the conditions. Similarly, Sil prevented extensively Bsep relocalisation, although a complete normalisation was not reached. Neither of the two protective agents modified the proportion of Bsep-associated fluorescence in the canalicular region.

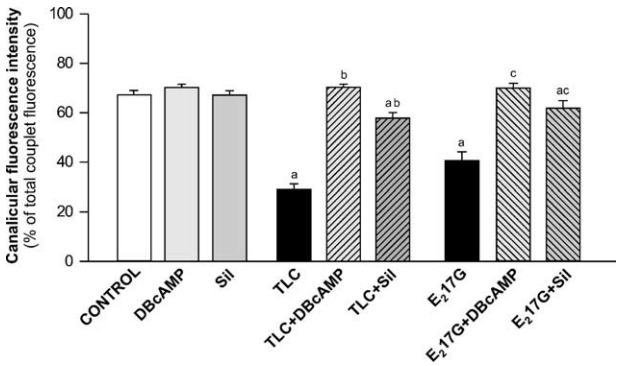

Fig. 2. Quantification of the canalicular Bsep-associated fluorescence in microphotographs equivalent to that depicted in Fig. 1. Bsep fluorescence intensity in the canalicular area was expressed as the percentage of total (canalicular membrane plus cell body) fluorescence intensity. Data represent mean ± S.E.M. of 15–20 microphotographs for each experimental group, randomly selected from experiments performed with three independent cellular preparations: (a) significantly different from control group ($p < 0.05$); (b) significantly different from TLC group ($p < 0.05$); (c) significantly different from $E_2$17G group ($p < 0.05$).

3.6. Effect of PKA inhibition and Ca^{2+} chelation on the effect of the different treatments over cVA of CLF

DBcAMP induces in hepatocytes both PKA activation and Ca^{2+} elevations [30,31]. The results of the studies aimed to assess which of these signalling molecules is involved in both DBcAMP and Sil anticholestatic effect are depicted in Fig. 3. The PKA inhibitors, KT5720 and H-89, and the Ca^{2+} chelator, BAPTA/AM, did not have any significant, independent effect on cVA of CLF (data not

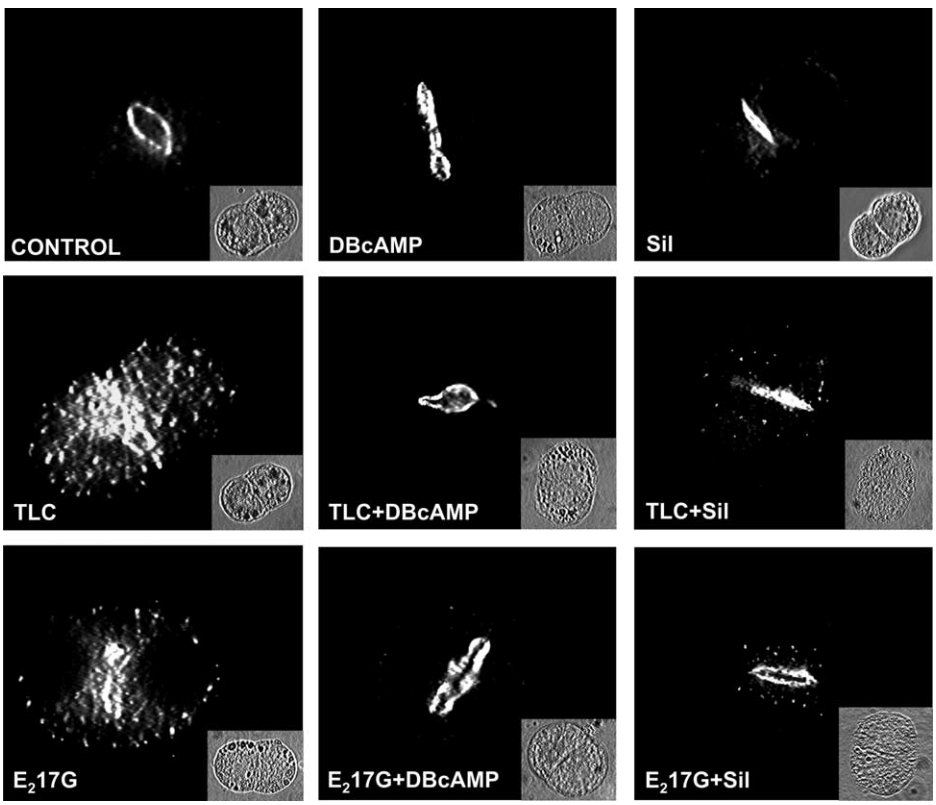

Fig. 1. Representative fluorescence microphotographs showing the effects of taurolithocholate (TLC; 2.5 μM, 20 min) or estradiol-17ß-D-glucuronide ($E_2$17G, 50 μM, 20 min) with or without pre-treatment with dibutyryl-cAMP (DBcAMP; 10 μM, 30 min) or silibinin (Sil, 2.5 μM, 30 min), on the localisation of Bsep in IRHC. Normal, canalicular localisation of Bsep is observed in control couplets, and neither DBcAMP nor Sil pre-treatments per se affected this distribution pattern. TLC and $E_2$17G induced endocytic retrieval of Bsep, visualized as numerous vesicular-like structures distributed in the whole cellular body. Preincubation of couplets with DBcAMP or Sil extensively prevents this phenomenon. Phase contrast microphotographs (insets) show that none of these treatments affected the normal couplet morphology.

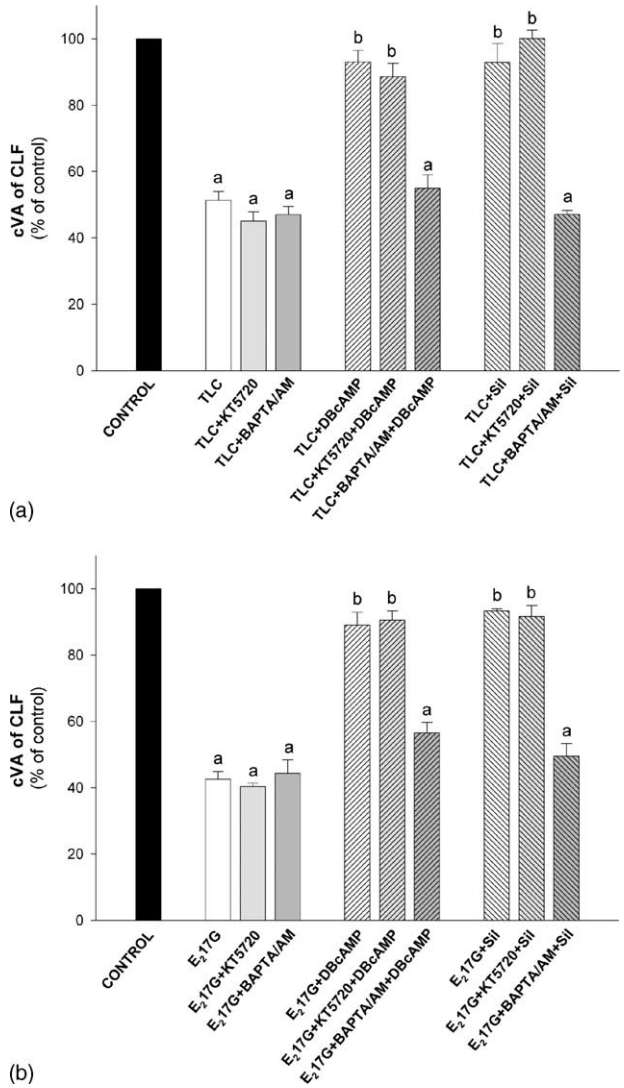

Fig. 3. Effect of inhibitors of protein kinase A (PKA) and Ca^{2+}-dependent signalling pathways on hepatoprotection afforded by dibutyryl-cAMP (DBcAMP) and silibinin (Sil) against taurolithocholate (TLC)- and estradiol-17ß-D-glucuronide ($E_2$17G)-induced decrease in canalicular vacuolar accumulation (cVA) of the fluorescent bile salt, cholyl-lylylfluorescein (CLF). Couplets were incubated for 15 min at 37 °C with dimethyl sulphoxide (DMSO, controls), BAPTA/AM or KT5720. Then, DBcAMP or Sil were added to the dishes, and cells were incubated for a further 30-min period. After removing Sil, couplets were exposed for 20 min to TLC (panel A) or $E_2$17G (panel B). cVA of CLF was then assessed as described in Section 2. All values are mean ± S.E.M., $n = 6$: (a) significantly different from control group ($p < 0.05$); (b) significantly different from TLC or $E_2$17G groups ($p < 0.05$).

shown). Similarly, in the absence of DBcAMP or Sil, these signalling modulators did not influence the ability of either TLC or $E_2$17G to impair cVA of CLF. Sil showed the same dependency on intracellular signalling mediators as DBcAMP to exert its protective effect, i.e., BAPTA/AM, but not the PKA inhibitor, KT5720, impaired the hepatoprotective effect of both the compounds. Lack of involvement of PKA-signalling pathway on the anticholestatic effect of both DBcAMP and Sil was confirmed by using another PKA inhibitor, H89, which had no effect on the capability of both hepatoprotectors to counteract the impairment of cVA of CLF induced by either TLC or $E_2$17G (data not shown).

4. Discussion

Recent studies from our group described the ability of silymarin to prevent the cholestasis induced by estrogens [17] and by the BS, TLC [18]. In both the circumstances, we concluded that improvement of canalicular transporter function plays a key role in silymarin effect, though the molecular mechanisms involved in its hepatoprotective properties remained unknown from these studies. The present study gains a deeper insight into this by demonstrating that Sil, the active component of silymarin, exerts its anticholestatic effect in $E_2$17G- and TLC-induced cholestasis, at least in part, by preventing the endocytic internalisation of the main canalicular BS transporter, Bsep. No protection against $E_2$17G-induced impairment on BS uptake was afforded by Sil, ruling out the possibility of a protective effect at the level of Ntcp function and/or localisation. Other possible mechanisms by which Sil prevents the cholestatic manifestations of both $E_2$17G and TLC can also be considered. For example, we have shown that silymarin significantly counteracted the impairment in canalicular transport function induced by chronic administration of 17α-ethynylestradiol without preventing the decrease in the number of the relevant transporters localised at this level [17]. This suggests that silymarin and, presumably, its active component, Sil, protect against alterations in the *intrinsic functional status* of the transporter as well, either by counteracting changes in the carrier itself or in its membrane environment. This mechanism could also be involved in the beneficial effects of silymarin on the BS transport impairment induced by $E_2$17G by mechanisms other than those involving endocytic internalization of Bsep, such as its putative *trans* inhibitory effect over this transporter [32]. Concerning TLC-induced alteration of BS transport, it has been described that chronic administration of lithocholate decreases expression of Bsep through farsenoid X receptor antagonist activity [33]. However, a role for this mechanism in our case is doubtful due to the acute nature of our model of TLC-induced cholestasis.

In theory, prevention of Bsep retrieval could be afforded either by inhibiting its endocytic internalisation or, alternatively, by stimulating its exocytic insertion into the canalicular membrane. It is currently thought that the second messenger, cAMP, prevents canalicular transporter retrieval in cholestasis [5,6,8] by stimulating the apically directed vesicular pathway; cAMP elevations lead to the insertion of several transport proteins into the canalicular membrane of the hepatocytes, including Bsep [34], Mrp2 [12], Mdr2 [34], Mdr1 [34], and AE2 [35].

The flavonolignans components of silymarin were characterized previously as potent cAMP phosphodiesterase inhibitors in an in vitro, cell-free protocol [19]. Here, we showed that Sil also exerts this effect in isolated hepatocytes, leading to an increase of approximately 40% in intracellular cAMP levels. Bearing in mind the above mentioned property of cAMP to improve BS transport function in cholestasis, this finding can be relevant to the mechanisms of silymarin-induced hepatoprotection. Indeed, cAMP may well function as a second messenger of Sil preventive effect on Bsep retrieval induced by cholestatic agents. Suggestively, Kurebayashi and Honda [36] showed that an increase in hepatic cAMP content induced by 16,16-dimethyl prostaglandin E_2 of similar magnitude to that induced by Sil in the present study was responsible, at least in part, for the hepatic protection against complement-mediated membrane damage. We extended here this concept by showing that elevations of intracellular cAMP are instrumental in preventing not only necrotic but also cholestatic injury.

cAMP acts as a second messenger in the liver by either activating PKA [37] or inducing elevations of cytosolic Ca^{2+} [30,31]. A comparison of the dependency on any of these signalling pathways of the anticholestatic properties of cAMP and Sil may afford a second line of evidence favouring cAMP as a second messenger of Sil hepatoprotective effects. Our results clearly showed that prevention by cAMP of TLC- and $E_2$17G-induced alteration of the BS canalicular transport in IRHC is a process dependent of Ca^{2+} mobilisation, but independent of PKA activation, and that Sil shared with cAMP this common signalling route. Selective dependency on Ca^{2+} elevation of cAMP anticholestatic effect in TLC-induced cholestasis had been previously reported by Milkiewicz et al. [29]. In the present study, we confirmed and extended this view in another cholestatic model, like that involving $E_2$17G. The consistent similarities between cAMP and Sil in both their anticholestatic mechanisms of action (i.e., prevention of Bsep endocytic internalisation) and the evoked signalling mediator (i.e., intracellular Ca^{2+}) adds support to the contention that the flavonolignan exerts its beneficial effect, at least in part, via this second messenger.

The mechanism by which Sil prevents, via cAMP-dependent Ca^{2+} elevations, the alterations in Bsep localisation and function induced by the cholestatic agents remains elusive from our results, but previous works by our group and others can provide some hints. Ca^{2+} elevations either using the Ca^{2+}-elevating compound, thapsigargin, or by administration of DBcAMP, stimulated canalicular transporter trafficking to the apical pole of IRHC during recovery of its polarity after isolation, in a Ca^{2+}-calmodulin-dependent fashion [13]. This most likely involves the capability of Ca^{2+} to stimulate hepatic vesicular-mediated transport, as suggested by the finding that the Ca^{2+}-elevating compounds, A23187 [38], vasopressin [39] and endothelin-1 [40], increase the second, transcytotic peak in the biliary excretion of the protein, horseradish peroxidase. Calmodulin involvement in Ca^{2+}-mediated stimulatory effect on this phenomenon is not surprising, since Ca^{2+}–calmodulin complex has been shown to be required in both microtubule-dependent and microtubule-independent (microfilament-dependent) transport steps in transcytosis, an effect probably exerted at the level of the actin-based and microtubule-based motor proteins, kinesin and myosin, respectively [41].

Summarizing, the present work provides evidence for a possible mechanism involved in the anticholestatic effects of silymarin: its main active component, Sil, is able to prevent the endocytic internalisation of Bsep, induced by TLC and $E_2$17G, in the IRHC model, improving the capacity of hepatocyte couplets to transport and accumulate into their canalicular vacuoles the fluorescent Bsep substrate, CLF. The molecular mechanism mediating Sil anticholestatic effect seems to involve, at least in part, cAMP as a second messenger, by utilizing downstream a Ca^{2+}-dependent intracellular signalling pathway.

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