# G-Protein-Coupled Receptor 30/Adenylyl Cyclase/ Protein Kinase A Pathway Is Involved in Estradiol 17ß-D-Glucuronide-Induced Cholestasis

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**Estradiol-17ß-D-glucuronide (E17G) activates different signaling pathways (e.g., $Ca^{2+}$-dependent protein kinase C, phosphoinositide 3-kinase/protein kinase B, mitogen-activated protein kinases [MAPKs] p38 and extracellular signal-related kinase 1/2, and estrogen receptor alpha) that lead to acute cholestasis in rat liver with retrieval of the canalicular transporters, bile salt export pump (Abcb11) and multidrug resistance-associated protein 2 (Abcc2). E17G shares with nonconjugated estradiol the capacity to activate these pathways. G-protein-coupled receptor 30 (GPR30) is a receptor implicated in nongenomic effects of estradiol, and the aim of this study was to analyze the potential role of this receptor and its downstream effectors in E17G-induced cholestasis. *In vitro*, GPR30 inhibition by G15 or its knockdown with small interfering RNA strongly prevented E17G-induced impairment of canalicular transporter function and localization. E17G increased cyclic adenosine monophosphate (cAMP) levels, and this increase was blocked by G15, linking GPR30 to adenylyl cyclase (AC). Moreover, AC inhibition totally prevented E17G insult. E17G also increased protein kinase A (PKA) activity, which was blocked by G15 and AC inhibitors, connecting the links of the pathway, GPR30-AC-PKA. PKA inhibition prevented E17G-induced cholestasis, whereas exchange protein activated directly by cyclic nucleotide/MAPK kinase, another cAMP downstream effector, was not implicated in cAMP cholestatic action. In the perfused rat liver model, inhibition of the GPR30-AC-PKA pathway totally prevented E17G-induced alteration in Abcb11 and Abcc2 function and localization. *Conclusion:* In conclusion, activation of GPR30-AC-PKA is a key factor in the alteration of canalicular transporter function and localization induced by E17G. Interaction of E17G with GPR30 may be the first event in the cascade of signaling activation. (HEPATOLOGY 2013; 00:000-000)**

Hepatocanalicular adenosine-triphosphate-dependent transporters are essential for bile secretion[1] and alteration in their expression, localization, or activity results in secretory failure and cholestasis.[2,3] Two of the most relevant canalicular transporters are the bile salt export pump (Abcb11; also named Bsep), which transports monoanionic bile salts, and multidrug resistance-associated protein 2

*Abbreviations: Ab, antibody; Abcb11,* bile salt export pump*; Abcc2,* multidrug resistance-associated protein 2*; AC, adenylyl cyclase; Akt, protein kinase B; cAMP, cyclic adenosine monophosphate; CGamF, cholyl-glycylamido-fluorescein; CMFDA, 5-chloromethylfluorescein diacetate; cPKC, $Ca^{2+}$-dependent PKC; 8-CPT, 8-CPT-2′-O-Me-cAMP; cVA, canalicular vacuolar accumulation; DMSO, dimethyl sulfoxide; DNP-G, S-(2,4-dinitrophenyl) glutathione; E17G, estradiol 17ß-D-glucuronide; EGFR, epidermal growth factor receptor; Epac, exchange protein activated directly by cyclic nucleotide; ER-α, estrogen receptor alpha; ERK, extracellular signal-related kinase; GPR30, G-protein-coupled receptor 30; GS-MF, glutathione methylfluorescein; IgG, immunoglobulin G; IRHCs, isolated rat hepatocyte couplets; IRT, initial rate of transport; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; nt, nucleotide; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PRL, perfused rat liver; siRNA, small interfering RNA; TC, sodium taurocholate.*

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*Received March 6, 2013; revised September 3, 2013; accepted September 16, 2013.*

*This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICTs 2006 no. 02012 and 2010 no. 1197) and Consejo Nacional de Investigaciones Cientificas y Técnicas (PIP 0691).*

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(Abcc2; also named Mrp2), which transports glutathione and glutathione conjugates, as well as a wide variety of anionic compounds, such as bilirubin glucuronides[1]; bile salts and glutathione are chief determinants of the so-called bile-salt-dependent and -independent fractions of bile flow, respectively.[4]

Different models of experimental cholestasis with clinical relevance, including estrogen-induced cholestasis, share characteristic alterations in the localization of canalicular transporters.[2,5,6] These works demonstrated that, in cholestatic conditions, Abcb11 and Abcc2 leave the canalicular membrane, undergoing endocytic internalization into vesicular compartments. This phenomenon was systematically associated with a failure in the secretion of their specific substrates, pointing to a key role of this pathomechanism in the cholestatic process.

In estrogen-induced cholestasis, and, in particular, in intrahepatic cholestasis of pregnancy, D-ring metabolites of estradiol may be a key factor in its pathogenesis. Their levels increase during pregnancy[7] and they induce acute and reversible cholestasis *in vivo* by impairing both fractions of bile flow.[8] Estradiol 17ß-D-glucuronide (E17G) is one of these endogenous D-ring metabolites. The mechanisms involved in E17G-induced cholestasis seem to be multifactorial. Transinhibition by E17G of Bsep-mediated canalicular transport of bile salts[9] has been shown to be a causal factor. In addition, our group showed that microtubule-independent endocytic internalization of both Abcb11[2] and Abcc2[10] is also a key cholestatic mechanism and a feature common to many other cholestatic conditions, both in experimental animals and humans.[11]

Intracellular signaling has emerged as a fundamental element to explain the development of different models of cholestasis.[12] In E17G-induced cholestasis, our group demonstrated that alteration in canalicular transporters depends on the activation of several signaling proteins, including "classical" ($Ca^{2+}$-dependent) protein kinase C (PKC) isoforms (cPKC),[13] phosphoinositide 3-kinase/protein kinase B (PI3K/Akt),[14] mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-related kinase (ERK)1/2,[15] and estrogen receptor alpha (ER-α).[16] Specifically, these previous works demonstrated that E17G activates, at least, two signaling branches to induce cholestatic alterations. The cPKC/ER-α-signaling pathway plays a critical role in the initial endocytic internalization of canalicular transporters that leads to cholestasis,[13,16] whereas PI3K/Akt is responsible for maintaining canalicular transporters internalized, which is independent of cPKC/ER-α activation.[13,14,16]

These actions of E17G are similar to the cytosolic (nongenomic) actions of nonconjugated estradiol described in different tissues, including liver.[17,18] G-protein-coupled receptor 30 (GPR30) has been implicated in the cytosolic effect of estradiol.[19,20] GPR30, a member of the G-protein-coupled receptor superfamily, is activated by estrogens and acts independently of ER-α to promote activation of the adenylyl cyclase/cyclic adenosine monophosphate (AC-cAMP)-dependent protein kinase A (PKA) pathway. Thus, GPR30 would play a critical role in rapid, nongenomic estrogen signaling.[20] One of the signaling molecules activated by GPR30 is epidermal growth factor receptor (EGFR).[20] Hence, the aim of this work was to evaluate the potential role of the GPR30-AC/cAMP-PKA pathway in E17G-induced cholestasis. Finally, we analyzed whether GPR30 plays a role in one of the previously characterized cholestatic signaling pathways, that is, cPKC/ER-α or PI3K/Akt, and the possibility that EGFR participates downstream of GPR30 in E17G-induced cholestatic alteration.

## Materials and Methods

Additional details on materials and methods are provided in the Supporting Materials.

***Isolated Rat Hepatocyte Couplet Treatments.*** Isolated rat hepatocyte couplets (IRHCs), obtained as previously described,[21] were exposed to E17G (50 μM) or dimethyl sulfoxide (DMSO; control) for 20 minutes, with or without pretreatment with inhibitors of the evaluated pathways.

The role of GPR30 in E17G-induced cholestasis was investigated by preincubating IRHCs with the specific GPR30 antagonist, G15 (10 nM), for 15 minutes before exposition to E17G. Then, to evaluate whether

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*View this article online at wileyonlinelibrary.com.*
*DOI 10.1002/hep.26752*
*Potential conflict of interest: Nothing to report.*
*Additional Supporting Information may be found in the online version of this article.*

J_ID: HEP Customer A_ID: HEP26752 Cadmus Art: HEP26752 Ed. Ref. No.: 13-0487.R2 Date: 1-October-13 Stage: Page: 3



specific activation of GPR30 was sufficient to reproduce E17G-induced cholestasis, cells were incubated only with the specific GPR30 agonist, G1 (1-1,000 nM), for 20 minutes.

The participation of AC in E17G-induced cholestatic alterations was examined by pretreating IRHCs with three different specific inhibitors of AC, SQ22,536 (SQ; 10 $\mu$M), MDL12,330 (MDL; 20 $\mu$M), or 2′3′-dideoxiadenosin (ddA; 1 $\mu$M), for 15 minutes. Activation of AC was evaluated measuring cAMP.[22]

The likely involvement of PKA in E17G-induced secretory failure was evaluated by incubating IRHCs with the PKA inhibitors, KT5720 (KT; 250 nM), H89 (1$\mu$M), or Rp-cAMPS (Rp; 10 $\mu$M), for 15 minutes. Activation of PKA was confirmed evaluating phospho-PKA substrates by western blotting.[23]

To evaluate the participation of the exchange protein activated directly by cyclic nucleotide (Epac) pathway in E17G-induced cholestatic alterations, experiments were performed under a condition of inhibition of the anticholestatic effects of Epac activation.[23] In these experiments, IRHCs were pretreated with colchicine (Colch; 1 $\mu$M) for 30 minutes and then exposed to the AC inhibitors, SQ (10 $\mu$M), MDL (20 $\mu$M), and ddA (1 $\mu$M), for 15 minutes, followed by incubation with 50 $\mu$M of 8-CPT-2′-O-Me-cAMP (8-CPT) for 15 minutes. Finally, IRHCs were treated with E17G. Activation of Epac was evaluated through phosphorylation of MAPK kinase (MEK; downstream Epac) by western blotting.[23]

Studies of GPR30 and ER-α/cPKC or PI3K coinhibition were carried out by coadministration of the antagonist, G15 (10 nM), together with either ER-α inhibitor ICI 182,780 (ICI; 1 $\mu$M), cPKC inhibitor Gö6976 (Gö; 1 $\mu$M), or PI3K inhibitor WM (100 nM) for 15 minutes before exposure to E17G (50 $\mu$M) for another 20 minutes.

Participation of EGFR in E17G-induced cholestatic alterations was evaluated by pretreating IRHCs with two different specific inhibitors of EGFR, Tyrphostyn AG1478 (TyrAG; 150 nM) and Cl-7387785 (Cl; 1 $\mu$M), for 15 minutes. Additionally, IRHCs were coincubated with TyrAG or Cl together with G15 or WM for 15 minutes, followed by exposure to E17G for another 20 minutes.

***Assessment of Abcb11 and Abcc2 Secretory Function and Localization in IRHCs.*** Functional changes in Abcb11 and Abcc2 under the treatments described above were evaluated by assessing the canalicular vacuolar accumulation (cVA) of cholyl-glycylamidofluorescein (CGamF), a fluorescent Abcb11 substrate (24),[24] or glutathione methylfluorescein (GS-MF), a fluorescent Abcc2 substrate derived from 5-chloromethylfluorescein diacetate (CMFDA),[25] as described previously.[5,23,25]

To evaluate the intracellular distribution of Abcb11 and Abcc2, IRHCs were treated with 200 $\mu$M of E17G, a concentration higher than that used in functional experiments, to render transporter retrieval more evident. After treatments, IRHCs were fixed, permeabilized, and incubated with a polyclonal antibody (Ab) against mouse Abcb11 or a monoclonal Ab against human ABCC2, followed by incubation with Cy2-conjugated donkey anti-rabbit immunoglobulin G (IgG) or Cy2-conjugated goat anti-mouse IgG, as described previously.[23] To delimit canaliculi, F-actin was stained by coincubating cells with Alexa Fluor 568 phalloidin (1:100; 1 hour).

***Synthesis of Small Interfering RNA.*** Four 21-nucleotide (nt) RNA duplexes (small interfering RNA; siRNA) targeting rat GPR30 messenger RNA were designed using the WIsiRNA selection program.[26] The control siRNA was designed by scrambling the nts of one of these specific targets. siRNAs were synthesized using Ambion's Silencer siRNA Kit (Ambion, Cambridge, MA).

***Assessment of Abcc2 Localization and Secretory Function in Hepatocytes Cultured in Collagen Sandwich.*** Isolated hepatocytes were treated with siRNA and cultured in collagen sandwich (SCRH) to allow a polarized configuration, as previously described.[16] After a culture period of 72 hours, SCRHs were treated with E17G (100 $\mu$M; 30 min) or vehicle (DMSO; control), and the functional status of Abcc2 was evaluated by determination of the pseudocanalicular accumulation of GS-MF, as previously described.[27] In brief, CMFDA was added to the medium and time-lapse imaging was done every minute during 8 minutes with a fluorescence microscope. Between 70 and 100 pseudocanaliculi were selected in each image, and the average of time fluorescence of GS-MF was measured. The slope of the line was estimated as a measure of initial rate of transport (IRT). Intracellular distribution of Abcc2 in SCRH after treatments was evaluated as previously described.[16]

## Results

***GPR30 Is Necessary, but Not Sufficient, for E17G to Induce Impairment of Canalicular Secretory Function.*** Figure 1A shows that pretreatment with the GPR30 antagonist, G15, almost completely prevented the effect of E17G on cVA of CGamF and GS-MF, thus indicating that GPR30 activation is necessary for E17G to produce its cholestatic effects. F1

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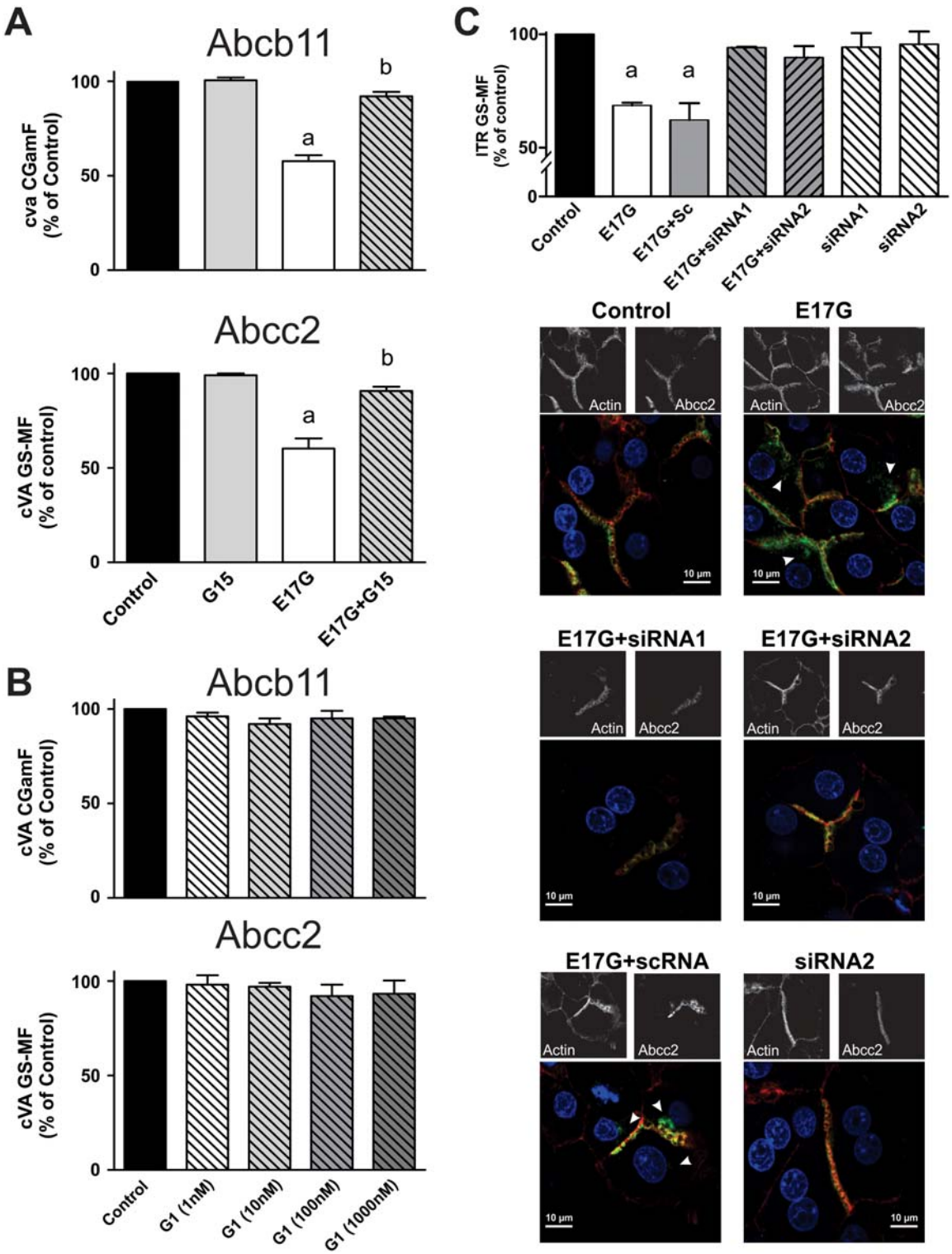


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Fig. 1. Prevention by GPR30 inhibition/knockdown of E17G-induced impairment of canalicular transporters Abcb11 and Abcc2. (A) IRHCs were preincubated with G15 (10 nM) for 15 minutes and then exposed to E17G (50 $\mu$M) for an additional 20-minute period preceding the addition of CGamF (0.3 $\mu$M) or CMFDA (2.5 $\mu$M) for 15 minutes. (B) IRHCs were incubated only with G1 (1-1,000 nM). cVA of CGamF and GS-MF was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to as control cVA values. cVA control values were 70% $\pm$ 2% for CGamF and 77% $\pm$ 1% for GS-MF. (C) SCRHs were transfected with two siRNAs for 48 hours and then exposed to E17G for 30 minutes. The slope of the curve obtained by graphing the average GS-MF-associated fluorescence of 70-100 pseudocanaliculi versus time was used to estimate the IRT (upper panel) of Abcc2. Representative confocal images are depicted (lower panels) showing cellular distribution of Abcc2 (green) in SCRH under different treatments. Actin network (red) and nuclei (blue) are also shown. Under control conditions, Abcc2-associated fluorescence is mainly localized at the canalicular membrane in the area delimited by the pericanalicular actin network. E17G induced a clear internalization of Abcc2-containing vesicles beyond the limits of the pericanalicular actin (indicated by arrowheads), a phenomenon significantly prevented by transfection with siRNA1 or siRNA2. None of the treatments affected the normal distribution of actin, which showed a predominant pericanalicular distribution. [a]Significantly different from control ($P < 0.05$); [b]significantly different from E17G and control ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (n = 3).

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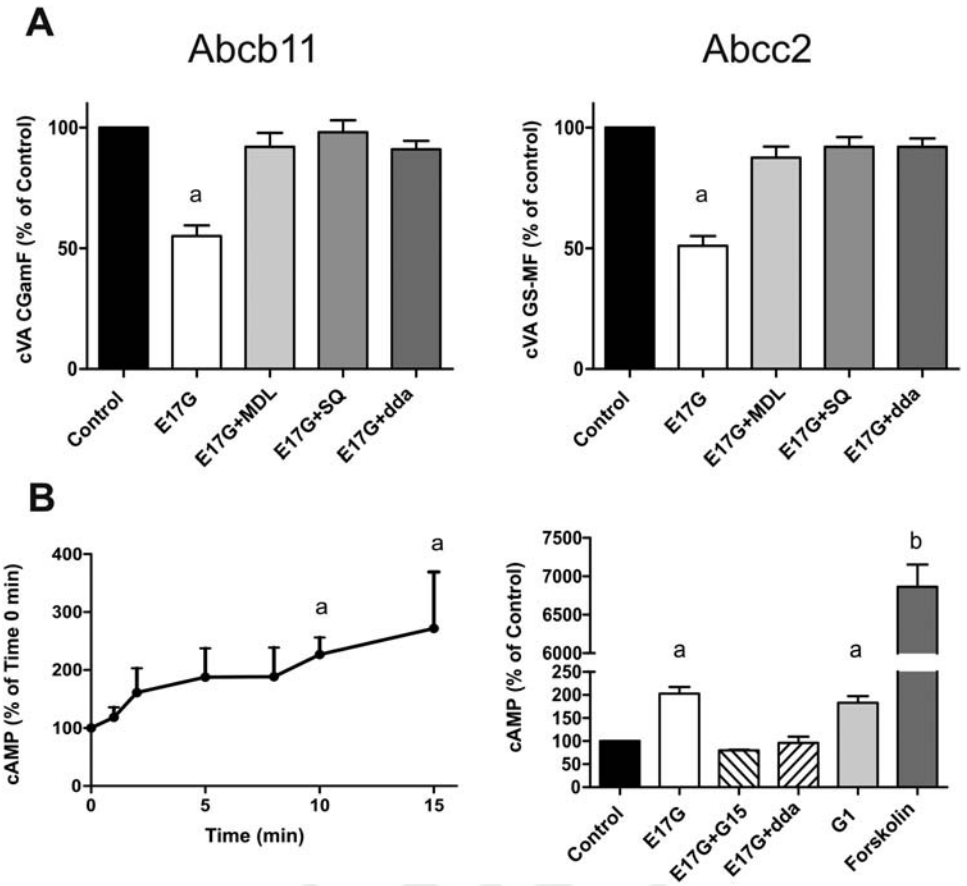


Fig. 2. Prevention by AC inhibition of E17G-induced impairment of cVA of CGamF or GS-MF. (A) IRHCs were preincubated with SQ (10 $\mu$M), MDL (20 $\mu$M), or ddA (1 $\mu$M) for 15 minutes and then exposed to E17G (50 $\mu$M) for an additional 20-minute period. Finally, cVA of CGamF (left panel) and GS-MF (right panel) was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to as control cVA values. cVA control values were 74% $\pm$ 3% for CGamF and 76% $\pm$ 3% for GS-MF. [a]Significantly different from control ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (SEM; n = 3). (B) Left panel shows temporal changes (15 minutes) of cAMP levels of IRHCs exposed to E17G (50 $\mu$M). Time 0 min = 0.8 $\pm$ 0.3 pmol/$10^5$ IRHC. [a]Significantly different from control at time = 0 min ($P < 0.05$). Right panel shows cAMP levels of IRHCs pretreated for 15 minutes with IBMX alone (0.8 mM; control), IBMX+G15 (10 nM), or IBMX+ddA (1 $\mu$M) and then exposed to E17G (50 $\mu$M), G1 (100 nM), or Forskolin (5 $\mu$M; positive control) for 10 minutes. Control = 1 $\pm$ 0.2 pmol/$10^5$ IRHC. [a]Significantly different from control ($P < 0.05$); [b]significantly different from control and E17G ($P < 0.05$). Results are expressed as percentage of control as mean $\pm$ SEM (n = 3).

Additionally, concentration-response experiments with the specific GPR30 agonist, G1, (Fig. 1B) show that this treatment did not modify cVA of CGamF and GS-MF, as compared to the control, thus indicating that the activation of GPR30 is necessary, but not sufficient, to produce cholestatic effects.

Participation of GPR30 in E17G-induced cholestatic alteration was confirmed by evaluation of function and localization of Abcc2 in SCRH transfected with siRNA1 and siRNA2. Figure 1C (upper panel) shows that GPR30 knockdown prevented the functional alteration induced by E17G. The same figure presents representative confocal images that show that E17G-induced internalization of Abcc2 was prevented by GPR30 knockdown, giving additional support to a role of GPR30 in E17G-induced actions. Cells treated with scrambled siRNA showed the same delocalization pattern of Abcc2 as E17G.

***AC Inhibition Prevents E17G-Induced Impairment of Canalicular Secretory Function.*** Figure 2A shows that treatment with AC inhibitors (SQ, MDL, or ddA) totally prevented the E17G-induced impairment in Abcb11 and Abcc2 activity, strongly suggesting that AC activation, and therefore cAMP synthesis, is required by E17G to exert its cholestatic effects. F2

To confirm the increase in cAMP levels induced by E17G, we incubated IRHCs with E17G in the presence of IBMX (phosphodiesterase inhibitor). Figure 2B shows that E17G increases cAMP, this increase being statistically significant after 10 minutes of incubation. At this time, the increase in cAMP levels induced by E17G had a magnitude similar to that

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attained by the GPR30 agonist, G1. In turn, the effects of E17G on cAMP levels were completely blocked when couplets were pretreated with G15 or ddA. These results indicate that E17G increases cAMP by GPR30-AC activation. To give additional support to the role of cAMP, Supporting Fig. 2 shows an experiment performed to make evident the procholestatic actions of the second messenger. This experiment also demonstrates that the presence of the cAMP analog, DB-cAMP, alone is not enough to produce the cholestatic effects.

***PKA, but Not Epac, Is Implicated in E17G-Induced Impairment of Canalicular Secretory Function.*** F3 Figure 3A shows that treatment with the three specific PKA inhibitors (KT, H89, or Rp) totally prevented E17G-induced impairment in Abcb11 and Abcc2 activities, thus indicating that PKA mediates E17G-induced cholestasis.

To confirm PKA activation by E17G, kinase activity was evaluated by western blotting of the phosphorylated forms of PKA substrates (Fig. 3B). As previously reported, these immunoblottings show several bands that would represent phosphorylated PKA substrates.[23,28,29] On the basis of their response to the PKA activator, DB-cAMP, and PKA inhibitors, two bands of 25 and 110 kDa were analyzed. E17G increased the phosphorylation of these two PKA substrates, and these increases were blocked by GPR30, AC, and PKA inhibitors.

Similarly to DB-cAMP, 8-CPT, a specific Epac agonist, has anticholestatic effects,[23] being likely that their procholestatic effects, if present, would be surpassed by its anticholestatic effects. As for DB-cAMP, the anticholestatic effects of 8-CPT depend on micro- F4 tubules[23] and may be blocked with Colch. Figure 4A shows that in conditions of inhibition of 8-CPT anticholestatic effects, this compound did not revert the preventive effects of AC inhibitors (MDL and ddA), thus indicating that Epac activation is not implicated in E17G-derived, cAMP-dependent cholestatic effects, at least by a microtubule-independent mechanism. In addition, western blottings of pMEK, an indicator of MEK activation (Epac downstream[30]), revealed that E17G does not affect MEK activation, whereas 8-CPT (positive control) significantly increased it (Fig. 4B).

***GPR30-AC-PKA Pathway Inhibition Prevents E17G-Induced Internalization of the Canalicular*** F5 ***Transporters, Abcb11 and Abcc2.*** Figure 5 shows confocal images of IRHC stained for Abcb11 (Fig. 5A) or Abcc2 (Fig. 5B) under different treatments. Confocal images show that, in control IRHCs, Abcb11 and Abcc2 were mainly localized within the actin pericanalicular ring space. IRHCs treated with G15, ddA, MDL, H89, or Rp alone exhibited the same distribution pattern (data not shown). E17G induced redistribution of both Abcb11 and Abcc2 over a greater distance from the center of the canalicular vacuoles. Pretreatment of IRHCs with GPR30, AC, and PKA inhibitors prevented this delocalization. These patterns of transporter distribution were confirmed by densitometric analysis of confocal images (Suppoiting Fig. 3). Moreover, densitometric analyses of actin distribution demonstrated that none of the treatments produced changes in the canalicular width.

***GPR30-AC-PKA Pathway Is Involved in the Impairment of Biliary Secretory Function Induced by E17G in Perfused Rat Liver.*** The bolus administration of E17G decreased bile flow to a minimum of approximately 38% of basal flow within 10 minuets, and bile flow did not recover throughout the perfusion period (Fig. 6A). This was accompanied by a decrease F6 in biliary excretion of the Abcc2 and Abcb11 substrates, S-(2,4-dinitrophenyl) glutathione (DNP-G; minimum, 45%; Fig. 6B) and sodium taurocholate (TC; minimum, 35%; Fig. 6C), respectively. Both transport activities recovered to approximately 70% of basal values from 15 minutes after E17G administration onward. The cumulative excretion of DNP-G decreased to 61% ± 3%, whereas TC cumulative excretion was 59% ± 2% of control values after E17G administration. Inhibition of GPR30-AC-PKA at three different levels prevented, in a similar manner, the initial drop in bile flow (minimum of approximately 78%) and completely prevented bile flow alterations induced by E17G from 15 minutes onward. G15, ddA, or H89 preadministration also preserved the biliary excretion of Abcc2 and Abcb11 substrates in E17G-treated rats, where DNP-G excretion reached a minimum of approximately 80% and that of TC reached a minimum of approximately 76%. Then, excretion of both substrates in rats treated with GPR30-AC-PKA inhibitors plus E17G increased and overtook substrate excretion in control rats 15 minuets after E17G injection, being similar to controls after. As a result, the cumulative excretion of DNP-SG (98% ± 2%) and TC (87% ± 5% of control) in G15/ddA/H89 plus E17G rats did not differ from that of control rats.

Confocal images show that in E17G-treated livers, both Abcb11 (Fig. 7A) and Abcc2 (Fig. 7B) were F7 detected in intracellular structures, consistent with their endocytic retrieval from the canalicular membrane. In livers perfused with G15/ddA/H89 plus

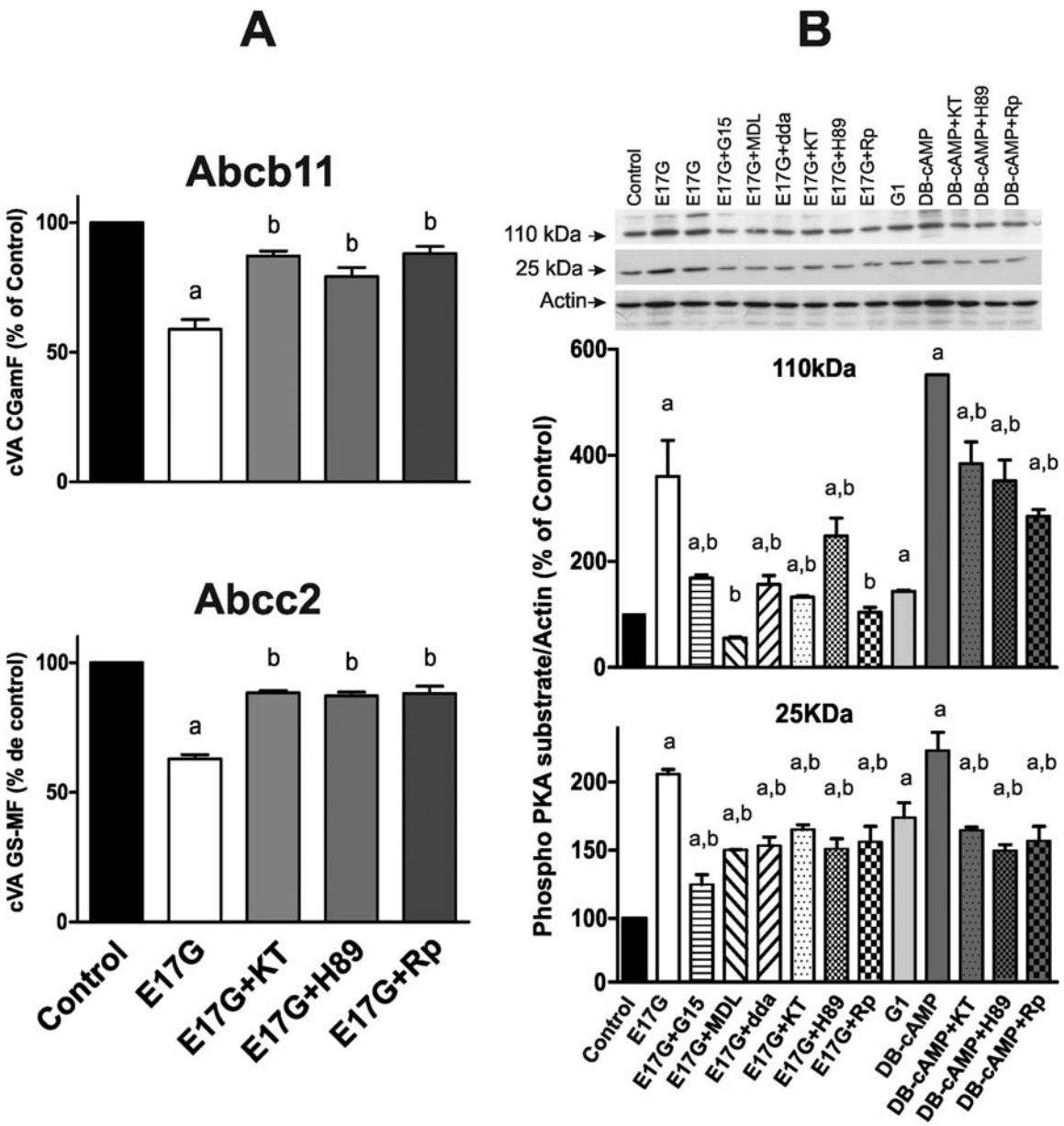


Fig. 3. Prevention by PKA inhibition of E17G-induced impairment of cVA of CGamF or GS-MF and estimation of PKA activation by E17G. (A) IRHCs were preincubated with KT (0.25 $\mu$M), H89 (1 $\mu$M), and Rp (10 $\mu$M) for 15 minutes and then exposed to E17G (50 $\mu$M) for an additional 20-minute period. Finally, cVA of CGamF (upper panel) and GS-MF (lower panel) was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to as control cVA values. cVA control values were 74% $\pm$ 3% for CGamF and 76% $\pm$ 3% for GS-MF. [a]Significantly different from control ($P < 0.05$); [b]significantly different from control and E17G ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (SEM; n = 3). (B) Isolated rat hepatocytes were incubated with DB-cAMP (10 $\mu$M; positive control) in the presence or absence of PKA inhibitors (KT [0.25 $\mu$M], H89 [1 $\mu$M], or Rp [10 $\mu$M]), G1 (100 nM), or E17G (50 $\mu$M) in presence or absence of GPR30-AC-PKA inhibitors (G15 [10 nM], MDL [20 $\mu$M], ddA [1 $\mu$M], KT [0.25 $\mu$M], H89 [1 $\mu$M], or Rp [10 $\mu$M]). PKA activity was determined by western blotting, using an Ab against phosphorylated PKA substrates. Two bands of 25 and 110 kDa were analyzed based on their response to DB-cAMP and PKA inhibitors. Two exposure times were necessary to reveal these bands: a short exposure for the 25-kDa band and a long exposure for the 110-kDa band. Differences in sample loading were corrected by the densitometric signal of the corresponding actin band. The ratio of each phosphorylated substrate/actin band density was compared to that of control bands (100%). Data are expressed as mean $\pm$ SEM (n = 3). [a]Significantly different from control ($P < 0.05$); [b]significantly different from the agonist alone ($P < 0.05$).

E17G, the distribution of both Abcb11 and Abcc2 was almost identical to that in control livers and this was confirmed by densitometric analysis (Supporting Fig. 4). Neither E17G nor GPR30-AC-PKA pathway inhibitors altered the canalicular width estimated by the two densitometric peaks of occludin.

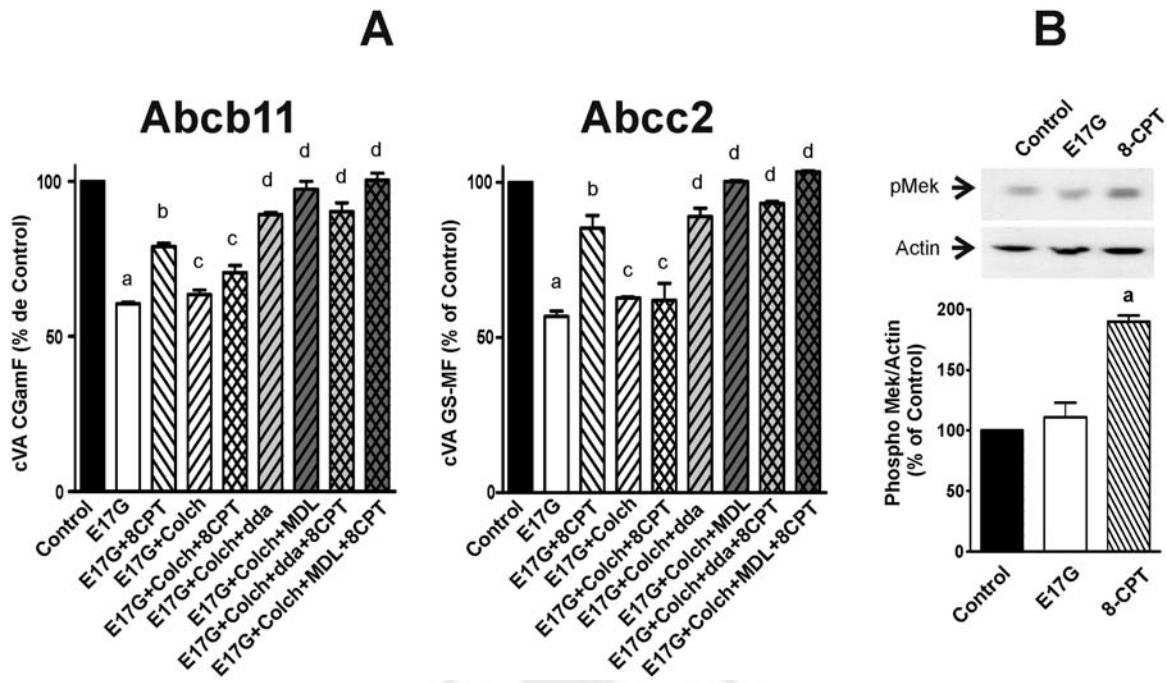


Fig. 4. Epac is not involved in E17G-induced alteration of canalicular transporters. (A) IRHCs were pretreated with Colch (1 $\mu$M) for 30 minutes and with MDL (20 $\mu$M), ddA (1 $\mu$M) for 15 minutes, followed by treatment with 8-CPT (50 $\mu$M) for 15 minutes and incubation with E17G (50 $\mu$M) for 20 minutes. Finally, IRHCs were exposed to CGamF (0.3 $\mu$M; left panel) and CMFDA (2.5 $\mu$M; right panel) for 15 minutes, and cVA of these fluorescent substrates was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to as control cVA values. Control cVA values were 74% $\pm$ 3% for CGamF and 75% $\pm$ 1% for GS-MF. [a]Significantly different from control ($P < 0.05$); [b]significantly different from E17G and control ($P < 0.05$); [c]significantly different from E17G+8-CPT ($P < 0.05$); [d]significantly different from Colch+E17G ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (SEM; n = 3). (B) Isolated rat hepatocytes were incubated with E17G (50 $\mu$M) and 8-CPT (50 $\mu$M; positive control). MEK (kinase downstream of EPAC) activity was determined by immunoblottings, using an Ab against phosphorylated MEK1/2. A band at 45 kDa was detected. Differences in sample loading were corrected by the densitometric signal of the corresponding actin band. The ratio of each pMEK/actin band density was compared with the ratio of control bands (100%). Data are expressed as mean $\pm$ SEM (n = 3). [a]Significantly different from control ($P < 0.05$).

***Interaction Between GPR30-AC-PKA and Other Signaling Pathways Implicated in E17G-Induced Cholestatic Alteration.*** The preventive effects of G15 and ICI or Gö on decreases in cVA of CGamF and GS-MF induced by E17G were additive (Fig. 8A), suggesting that GPR30 and cPKC/ER-$\alpha$ were acting independently. This was confirmed in cholestatic alterations induced by E17G because neither ICI affects the increase of cAMP produced by E17G (Supporting Fig. 5A, left panel) nor does treatment with G15, ddA, or H89 (Supporting Fig. 5A, right panel) affect the activation of ER-$\alpha$ measured by western blotting.

Contrarily, no additive effect was observed with G15 and WM (Fig. 8A), suggesting that GPR30 and PI3K share a common pathway. The sequential activation of GPR30 and PI3K was confirmed by measuring the activation of Akt, a kinase downstream PI3K. Figure 8B shows that pretreatment with G15 prevented the activation of Akt produced by E17G. Moreover, PI3K inhibition did not affect the increase in cAMP produced by E17G (Supporting Fig. 5A, left panel). However, ddA and H89 did not affect Akt activation. These findings indicate that PI3K/Akt activation is mediated by GPR30 independently of AC or PKA.

Finally, we analyzed the participation of EGFR in E17G-induced cholestasis by pretreatment of IRHCs with two specific inhibitors of EGFR (TyrAG and Cl). Both inhibitors partially prevented the decrease in cVA of CGamF and GS-MF induced by E17G, indicating that EGFR participates in the E17G cholestasis. We found additive-preventive effects when cells were coincubated with G15 or WM, suggesting that EGFR participates in E17G-induced cholestasic alteration by a mechanism independent of GPR30/PI3K. This independent action was confirmed because neither EGFR inhibitors affected the increase in cAMP levels (Supporting Fig. 5A, left panel) or Akt activation induced by E17G (Fig. 8C) nor did G15, ddA, H89, and WM affect the activation of EGFR produced by E17G (Supporting Fig. 5B). These data show that EGFR is activated, and participates, in E17G-induced cholestasis through a mechanism independent from GPR30, AC, PKA, and PI3K.

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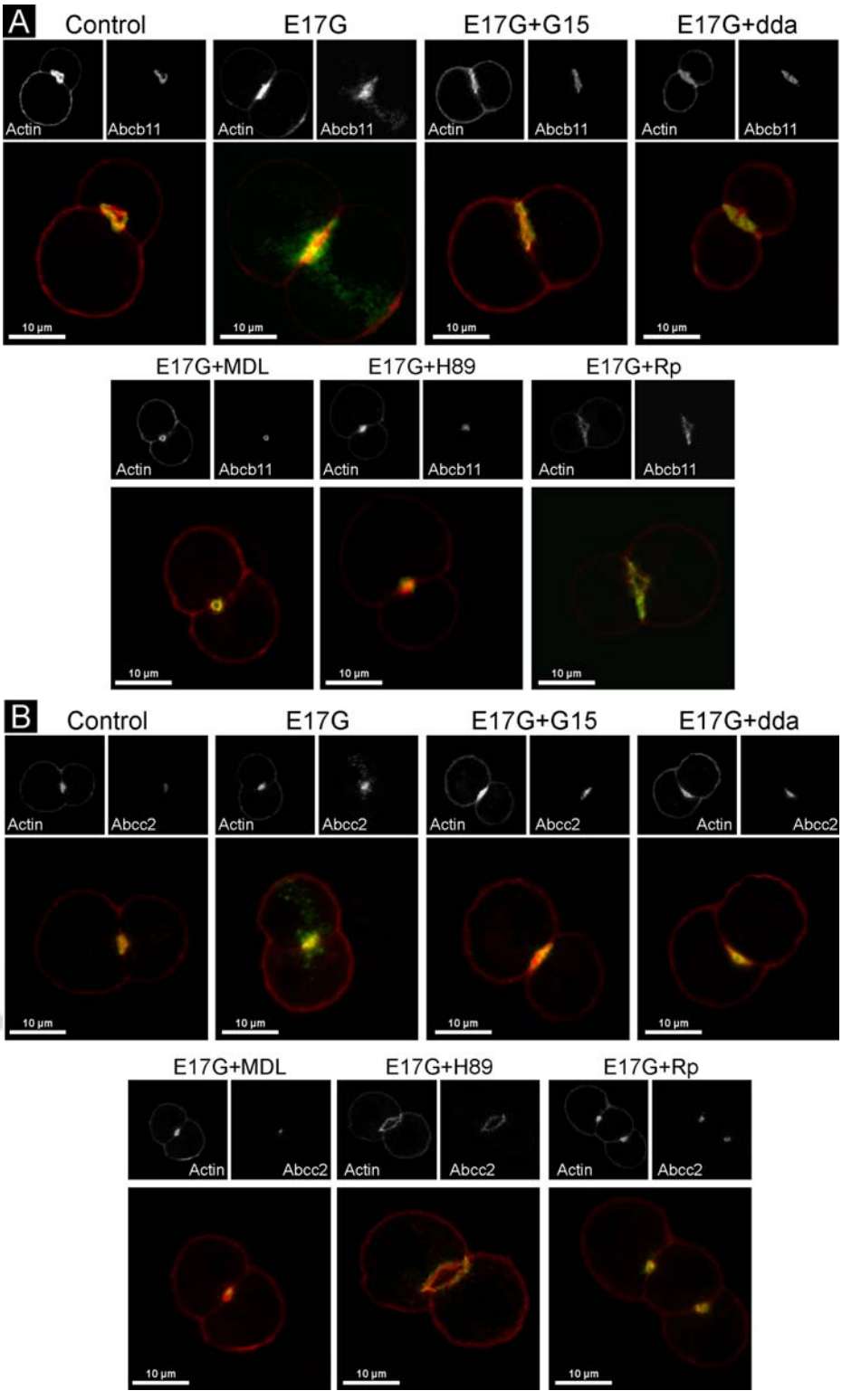


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Fig. 5. Prevention of E17G (200 $\mu$M)-induced retrieval of Abcb11/Abcc2 by inhibition of the GPR30-AC-PKA pathway. Representative confocal images show cellular distribution of Abcb11 (A) or Abcc2 (B), actin, and merged images in IRHCs under different treatments. Under control conditions, Abcb11/Abcc2-associated fluorescence is mainly localized at the canalicular membrane in the area delimited by the pericanalicular actin ring. E17G induced a clear internalization of Abcb11/Abcc2-containing vesicles beyond the limits of the pericanalicular actin ring, a phenomenon significantly prevented by treatment of IRHC with G15 (10 nM), MDL (20 $\mu$M) ddA (1 $\mu$M), H89 (1 $\mu$M), or Rp (10 $\mu$M). None of the treatments affected the normal distribution of actin, which showed a predominant pericanalicular distribution.

## Discussion

Intracellular signaling cascades are key factors in the onset of cholestatic phenomena.[12] In E17G-induced cholestasis, several signaling molecules have been implicated, such as ER-$\alpha$, cPKC, and PI3K.[13-16] In the present study, we demonstrate the participation of the cAMP-PKA pathway in estrogen cholestasis. The association of this pathway with E17G effects was revealed by the strong anticholestatic actions associated with inhibition of adenylyl-cyclases and PKA.

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Generally, cAMP synthesis starts with stimulation of a G-protein-coupled receptor and, consequently, with the activation of an adenylyl-cyclase.[31] GPR30 is a receptor that mediates several cytosolic effects of nonconjugated estradiol in several tissues, including the liver.[19] Given that nonconjugated estradiol and E17G activate similar pathways,[13-16,18,31] we analyzed whether E17G is able to mediate its cholestatic effect by GPR30 activation. We observed that blocking GPR30, by inhibition or knockdown, prevented, almost completely, the cholestatic effects induced by E17G. We also proved that E17G-induced increase in cAMP levels or PKA activation are completely blocked in the presence of G15, an antagonist of GPR30. This finding points to a sequential activation of the GPR30-AC-PKA pathway. In line with this, Hsieh et al.[32] showed PKA activation by nonconjugated estradiol by GPR30 modulation. Furthermore, we observed that another potential effector pathway of cAMP, that of the Epac protein, is not involved in E17G-induced cholestasis.

It is worth noting that specific activation of GPR30 using the agonist G1 or direct administration of DB-cAMP, under a condition of inhibition of its anticholestatic effects, did not reproduce the cholestatic effect induced by E17G. Potential explanations for these findings could be that the action of E17G on GPR30 evokes different downstream signaling pathways, as compared to activation by the specific agonist, G1 (e.g., mediated by post-translational modifications of GPR30 by E17G) or, alternatively, that E17G needs to modulate additional signaling molecules to induce cholestasis. A potential candidate associated with this fact is ER-α. Indeed, our group recently demonstrated a critical role of the ER-α/cPKC-signaling pathway in the initial endocytic internalization of canalicular transporters induced by E17G.[13,16] In this work, we give evidences indicating that GPR30 and cPKC/ER-α act in different pathways, so we can speculate that E17G activates GPR30 and simultaneously modulates ER-α activity, these two events being necessary and independent to produce cholestasis.

Recently, our group also showed that E17G activates a pathway that involves PI3K/Akt, allegedly responsible for maintaining internalized canalicular

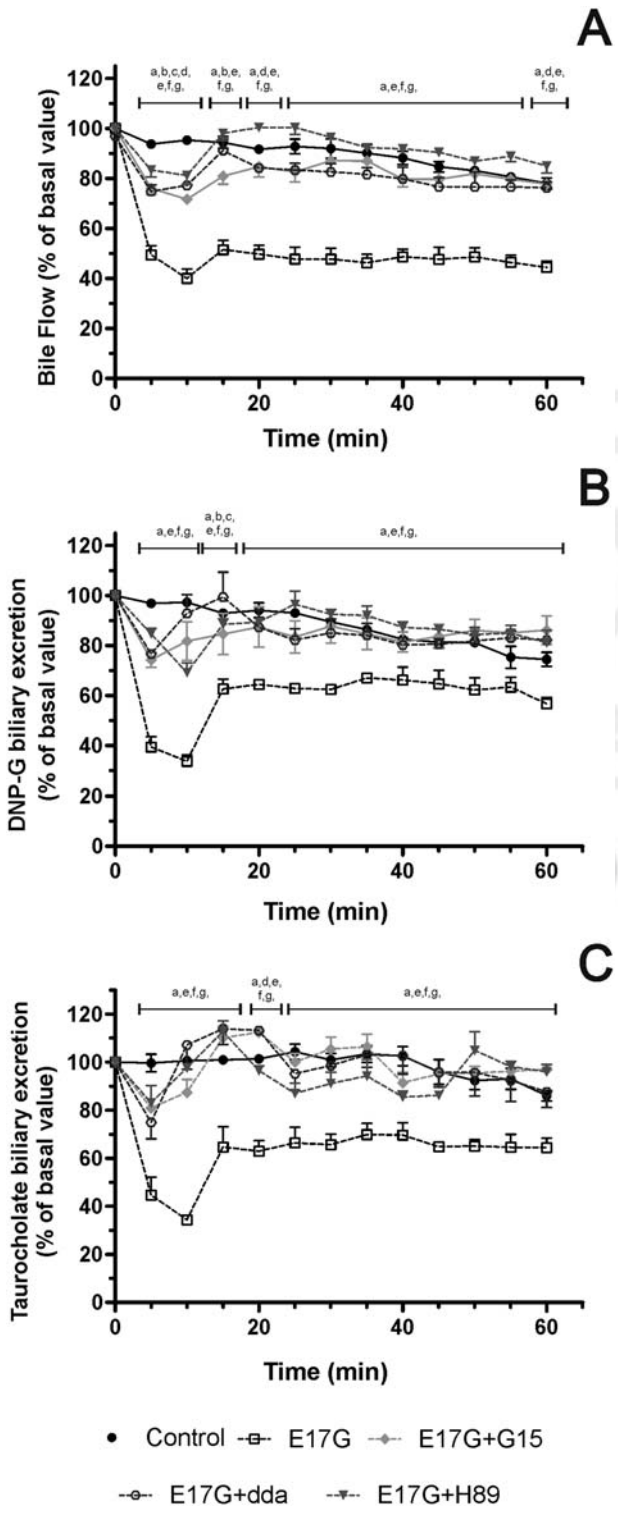


Fig. 6.

Fig. 6. Inhibition of the GPR30-AC-PKA pathway protects against E17G-induced impairment of bile flow and biliary secretion of DNP-G and TC in PRL. Graphics show temporal changes in bile flow (A) and in the biliary excretion rate of both total DNP-G (B) and TC (C) throughout the perfusion period. PRLs were treated with a bolus of E17G (3 μmol/liver) or its vehicle (DMSO/bovine serum albumin 10% in saline; control) in the presence or absence of G15 (10 nM), ddA (1 μM), or H89 (1 μM). [a]Control significantly different from E17G; [b]E17G+G15 significantly different from control; [c]E17G+ddA significantly different from control; [d]E17G+H89 significantly different from control; [e]E17G+G15 significantly different from E17G; [f]E17G+ddA significantly different from E17G; [g]E17G+H89 significantly different from E17G ($P < 0.05$; n = 3 animals per group).

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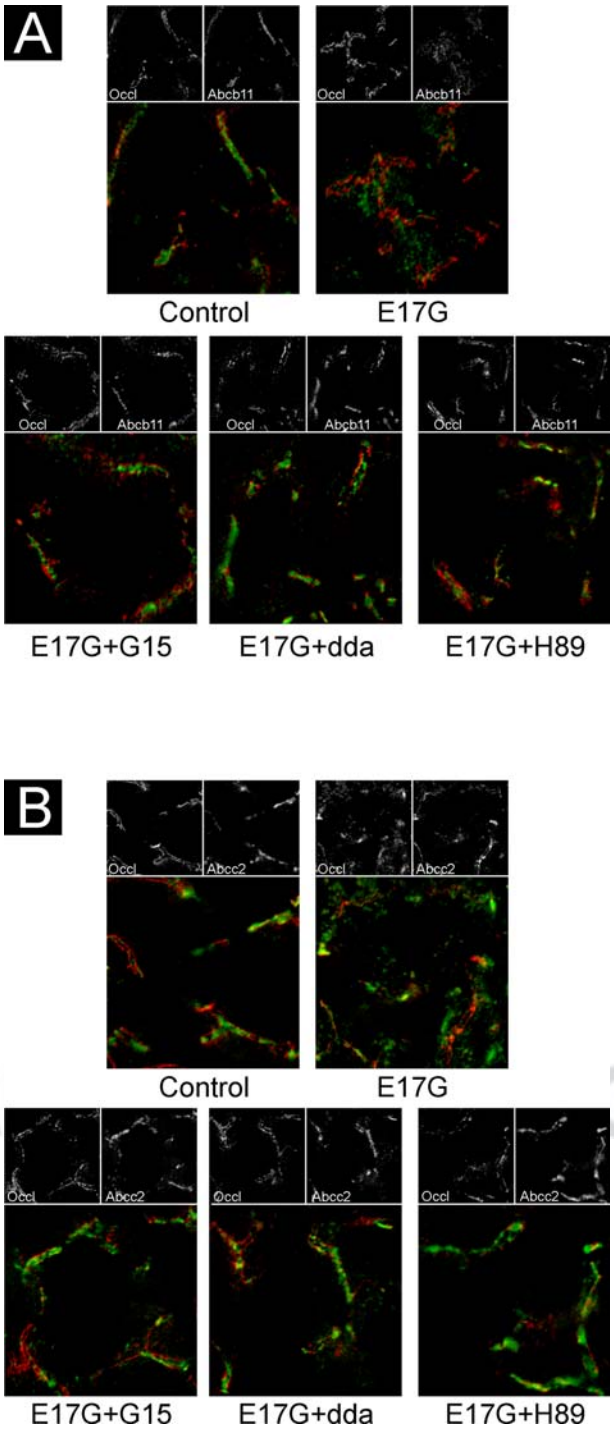


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Fig. 7. Inhibition of the GPR30-AC-PKA pathway prevents E17G-induced retrieval of Abcb11 (A) and Abcc2 (B) in PBL. Representative confocal images of immunostained liver samples show distribution of Abcb11 (A) or Abcc2 (B) and occludin. In control livers, both Abcb11 and Abcc2 were mainly confined to the canalicular space delineated by the tight junction-associated protein, occludin. After E17G (3 $\mu$mol/liver), some canaliculi show intracellular fluorescence associated with Abcb11 or Abcc2 at a greater distance from the canalicular membrane, consistent with their endocytic retrieval. G15 (10 nM), ddA (1 $\mu$M), or H89 (1 $\mu$M), 15 minutes before E17G addition, prevented the internalization of canalicular transporters, as illustrated by a control-like pattern of Abcb11 and Abcc2 distribution. G15, ddA, or H89 themselves did not induce any changes in transporter localization.

transporters, which is independent of cPKC/ER activation.[13,16] These works demonstrated that inhibition of either of these two signaling pathways had a partial preventive effect. The results obtained in the present study also show, both in IRHC and perfused rat liver (PRL) models, that inhibition of the GPR30/AC/PKA

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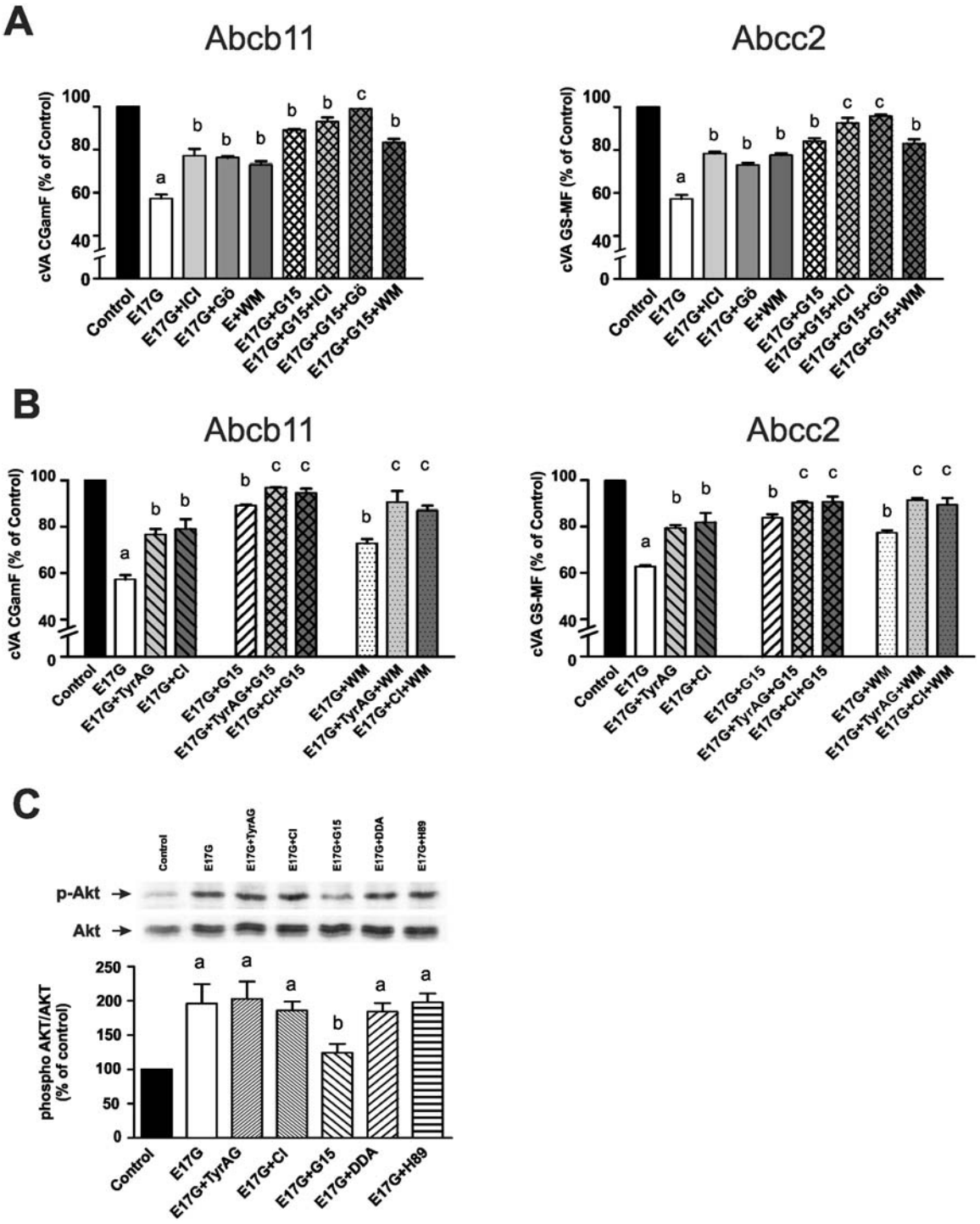


Fig. 8. Possible mechanisms by which GPR30-AC-PKA participates in E17G-induced canalicular secretory failure. (A) IRHCs were treated with G15 (10 nM) together with ICI (1 $\mu$M), Gö (1 $\mu$M), or WM (100 nM) for 15 minutes, followed by treatment with E17G (50 $\mu$M) for 20 minutes. Finally, IRHCs were exposed to CGamF (0.3 $\mu$M; upper panel) and CMFDA (2.5 $\mu$M; lower panel) for 15 minutes, and cVA of these fluorescent substrates was calculated as the percentage of couplets displaying visible fluorescence in their canalicular vacuoles from a total of at least 200 couplets per preparation, referred to as control cVA values. Control cVA values were 70% $\pm$ 3% for CGamF and 71% $\pm$ 1% for GS-MF. [a]Significantly different from control ($P < 0.05$); [b]significantly different from E17G and control ($P < 0.05$); [c]significantly different from E17G+G15, E17G+ICI, E17G+Gö, and E17G+WM ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (SEM; n = 3). (B) IRHCs were treated with TyrAG (150 nM) or Cl (1 $\mu$M) together with G15 (10 nM) or WM (100 nM) for 15 minutes, followed by treatment with E17G (50 $\mu$M) for 20 minutes. Finally, IRHCs were exposed to CGamF (0.3 $\mu$M; upper panel) and CMFDA (2.5 $\mu$M; lower panel) for 15 minutes and cVA of these fluorescent substrates was calculated. Control cVA values were 69% $\pm$ 1% for CGamF and 68% $\pm$ 3% for GS-MF. [a]Significantly different from control ($P < 0.05$); [b]significantly different from E17G and control ($P < 0.05$); [c]significantly different from E17G+TyrAG, E17G+Cl, E17G+G15, and E17G+WM ($P < 0.05$). Data are expressed as mean $\pm$ SEM (n = 3). (C) Isolated rat hepatocytes were preincubated with G15 (10 nM), ddA (1 $\mu$M), or H89 (1 $\mu$M), WM (100 nM), TyrAG (150 nM), and Cl (1 $\mu$M) for 15 minutes, followed by incubation with E17G (200 $\mu$M) for 20 minutes. PI3K activation was determined using an Ab against phosphorylated Akt (a PI3K effector). A band at 56 kDa was detected. The ratio of pAkt/total Akt density for each treatment was compared to the ratio of control bands (100%). Data are expressed as mean $\pm$ SEM (n = 3). [a]Significantly different from control; [b]significantly different from E17G ($P < 0.05$).

pathway at any of its three levels produces a strong preventive effect on E17G-induced impairment of transporter activity and localization. These results allow us to speculate that this signaling pathway would play a pivotal role in initiating E17G-induced cholestasis. Studies of the interdependence of the PI3K/Akt and GPR30 pathways indicate that E17G activates PI3K/Akt through GPR30, but independently of AC or PKA. These findings allow us to speculate that GPR30 can activate at least two signaling branches: one dependent and the other independent of AC/PKA.

The pathway that relies on cAMP and PKA activation could play a role in the endocytosis of canalicular transporters independent from the ER-α/cPKC pathway. In line with this, the dual anchoring protein, D-AKAP2/AKAP10, a binding partner for both the RI and RII subunits of PKA, interacts with Rab11 and promotes accumulation of recycling transferrin in Rab11-positive endosomes.[33] Because internalized Abcb11 and Abcc2 colocalize with Rab11a,[23] it is possible to speculate on a role for PKA/AKAP in the recycling of endosomes containing Rab11a and Abcb11 or Abcc2.

Another possible target of the cAMP/PKA pathway is the regulation of the calcium/calmodulin pathway. We have recently provided evidence that L-type calcium channels, calmodulin, and a direct effector (CamKII) participate in E17G-induced cholestasic alterations.[34] In relation with this, PKA can regulate L-type calcium channels[35] and phosphorylate ryanodine receptor, thus modulating intracellular calcium levels.[36] Moreover, PKA can modulate endocytosis in neurons by calmodulin activity modulation.[37] Hence, it is possible to hypothesize that activation of PKA by E17G triggers calcium/calmodulin-dependent signaling that leads to endocytosis of Abcb11 and Abcc2.

The other signaling branch triggered by GPR30 could activate the PI3K/Akt pathway and maintain internalized Abcb11 and Abcc2. Consistent with these results, Wang et al.[20] propose that estrogen regulates a plethora of biological processes through the membrane-initiated signaling of the GPR30 pathway, in which PI3K would act downstream of this receptor by a mechanism independent of AC/PKA. In this work, transactivation of EGFR by GPR30 was also described as a common mechanism that finally activates different effectors (including PI3K). So, we analyzed the participation of EGFR in E17G-induced cholestasis. We observed both activation of EGFR by E17G and prevention of cholestasis by EGFR inhibitors. However, neither was EGFR activation blocked by G15 nor did experiments with coinhibition of GPR30 or PI3K and EGFR show nonadditive effects. These indicate that EGFR participates in E17G-induced cholestasis by a mechanism independent of GPR30. Song et al.[38] described another mechanism by which EGFR participates in extranuclear effects of nonconjugated estradiol. The mechanism proposed is that classical ER-α transactivates EGFR and this initiates nongenomic signaling pathways. Our results and this fact allow us to speculate that in E17G-induced cholestasis, EGFR is probably activated by ER-α, independently of GPR30.

Finally, this work represents the first report where cAMP behaves as a procholestatic signaling molecule promoting endocytic internalization of canalicular transporters. In contrast to these results, numerous studies have previously demonstrated that cAMP is an anticholestatic signaling molecule.[23,39] These opposing effects of cAMP can be explained by the compartmentalization of this second messenger, because within the cell, adenylyl-cyclases, phosphodiesterases, and pathway effectors are closely associated. Therefore, cAMP generation and degradation is compartmentalized, leading to the existence of localized pools of the messenger. In accord with this fact, our group recently showed that cAMP derived from hormones, such as glucagon and adrenaline, have beneficial effects by the activation of different intracellular mechanisms.[11,23] Further supporting this, experiments performed in cellular fractions (cytosolic, mitochondrial, and plasmatic membrane fractions) showed that the pattern of phosphorylation of PKA substrates differs between glucagon and E17G (Supporting Fig. 6). In cytosolic fractions, we found a band of 80 kDa that is phosphorylated mainly by E17G, but not by glucagon, and another of 110 kDa that is phosphorylated by both. Instead, in mitochondrial fractions, a band at 37 kDa is strongly phosphorylated by glucagon and, to a lesser extent, by E17G, and a band of 25 kDA is phosphorylated by both. In plasmatic membrane fraction, we found a possible substrate at 50 kDA that is phosphorylated in a similar manner by Glu and E17G.

In summary, activation of GPR30-AC-PKA is a key factor in the alteration of canalicular transporters induced by E17G. The interaction of E17G with GPR30 would be the first event in the cascade of signaling activation. This hypothesis is supported by the fact that GPR30 or AC inhibition completely blocked E17G insult, a feature that differs with what was previously observed in experiments using PKC and PI3K inhibitors, whose protection was only partial. Nevertheless, GPR30-AC-PKA activation is necessary for E17G-induced cholestasis, but is not enough, because

the agonists of this pathway, when used alone, were not able to trigger the cholestatic process.

*Acknowledgments:* The authors express their gratitude to Dr. Marcelo G. Luquita for his skillful contributions with HPLC determinations and to Mr. Diego R. Taborda for his helpful assistance with animal procedures and cell isolation.

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