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Hepatocanalicular organic-anion transport is regulated by protein kinase C

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In order to investigate the regulation of canalicular organic-anion transport, we used a hepatocyte transport assay in which canalicular secretion of a model organic anion, dinitrophenyl-glutathione (GS-DNP), was measured in the presence of stimulators and inhibitors of the Ca^{2+}/protein kinase C (PKC) second-messenger system and of the cyclic AMP (cAMP) second-messenger system. Vasopressin (24 nM) and the phorbol ester phorbol 12-myristate 13-acetate (1 μg/ml), both stimulators of PKC, stimulated GS-DNP efflux by 65 ± 36% and 55 ± 28% respectively, whereas staurosporine (10 μM), an inhibitor of PKC, inhibited efflux by 53 ± 13%. Glucagon and forskolin, both stimulators of the cAMP second-messenger system, as well as the cAMP analogue dibutyryl cAMP and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, did not significantly influence the GS-DNP efflux. It can be concluded that canalicular organic-anion transport in hepatocytes is either directly or indirectly regulated by PKC.

INTRODUCTION

We have previously characterized a transport system for organic anions in the canalicular membrane of hepatocytes, which we have termed Multispecific Organic Anion Transporter (MOAT). This transporter concentrates a number of organic anions into bile, and it was demonstrated that transport is ATP-dependent [1]. It could be demonstrated, with both isolated hepatocytes [2] and vesicular plasma-membrane preparations [3,4], that the MOAT is not functional in the TR^- rat, a mutant Wistar rat strain with inherited conjugated hyperbilirubinaemia [5]. A wide range of organic anions is not, or is very poorly, secreted into the bile of the TR^- rat. These anions include glutathione conjugates such as 2,4-dinitrophenyl-*S*-glutathione (GS-DNP) and tetrabromosulphophthalein-glutathione (GS-BSP) [2], but also more physiological substrates such as conjugated bilirubin [6], GSSG [2] and cysteinyl-leukotrienes [7]. Also, the secretion of some bile acids conjugated at the 3-position with glucuronide or sulphate is strongly impaired in the TR^- rat [8], whereas the transport of univalent bile acids such as taurocholate is not impaired [9]. These observations suggest that substrates for the MOAT should contain at least two negatively charged groups, separated by a more or less hydrophobic region in the molecule [8].

So far the regulation of this hepatocanalicular organic-anion-transporting system has not been investigated. Functionally, MOAT resembles the P-glycoprotein, a protein that is associated with the multi-drug-resistance (MDR) phenotype [10]. Both systems pump a wide variety of organic compounds out of the cell at the direct expense of ATP. Both transport systems are specifically present in the canalicular membrane of the hepatocyte. However, P-glycoprotein seems to recognize cationic and neutral compounds, whereas MOAT is specific for anions. The MDR system was reported to be regulated by phosphorylation through the protein kinase C (PKC) pathway [11,12]. This led us to the hypothesis that PKC could also be important in the regulation of organic-anion transport.

To study whether MOAT is coupled to a second-messenger system, an assay was used by which canalicular transport of GS-DNP in isolated hepatocytes can be studied [2]. We show that the efflux rate of GS-DNP from these cells increases if the cells are stimulated with vasopressin (VP) or the phorbol ester phorbol 12-myristate 13-acetate (PMA), both activators of PKC. Staurosporine, an inhibitor of PKC, decreases the GS-DNP efflux rate, whereas modulators of the cyclic AMP (cAMP) second-messenger system do not influence the GS-DNP efflux rate. This suggests a role for PKC in the regulation of canalicular organic-anion transport in the liver.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Janssen (Beerse, Belgium). ^{14}C-labelled CDNB (sp. radioactivity 5.87 mCi/mmol) was obtained from Amersham (Houten, The Netherlands). Silicone oil was from Wacker Chemie (Munich, Germany). PMA, [Arg]VP, forskolin, glucagon, 3-isobutyl-1-methylxanthine (IBMX), N^6,2′-*O*-dibutyryl cyclic AMP (sodium salt) (db-cAMP), phenylmethanesulphonyl fluoride and leupeptin were all obtained from Sigma (St. Louis, MO, U.S.A.). Staurosporine was obtained from Boehringer (Mannheim, Germany). The PKC assay system was from Amersham. DEAE-Sephacel was from Pharmacia.

Animals

Normal male Wistar rats, weighing approx. 300 g, were obtained from Harlan-CPB, Zeist, The Netherlands. Male TR^- mutant rats of the same weight came from our own breeding colonies. This strain has been characterized [3,5,8,9]. Animals were maintained on normal laboratory chow and had free access to water. All experiments were carried out with fed rats.

Abbreviations used: GS-DNP, 2,4-dinitrophenyl-*S*-glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GS-BSP, tetrabromosulphophthalein-glutathione; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; VP, vasopressin; IBMX, 3-isobutyl-1-methylxanthine; cAMP, cyclic AMP; MOAT, multispecific organic-anion transporter; GST, glutathione *S*-transferase; MDR, multi-drug-resistance.

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Preparation of hepatocytes

Hepatocytes were isolated by the method of Berry & Friend [13], modified as described in [2].

GS-DNP transport assay

GS-DNP efflux was measured as described previously [1,2]. Briefly, hepatocytes (10 mg dry wt./ml) were preincubated in Krebs bicarbonate buffer [2] at 37 °C for 20 min, and then the hormone was added for 3 or 10 min (as indicated in the Tables and Figures). Subsequently a mixture of [^{14}C]CDNB (0.5 μmol/g dry wt.) and unlabelled CDNB (2.5 μmol/g dry wt.) was presented to the cells. Samples were taken at 30, 90 and 150 s after addition of CDNB, and cells were separated from the medium by immediate centrifugation (30 s, 10000 *g*) through a silicone-oil layer into 10% $HClO_4$. Radioactivity was determined in the upper (medium) fraction. Under the conditions described, secretion of GS-DNP was always linear ($r > 0.99$ by linear regression). Previously we have shown that the uptake and conjugation of CDNB is complete within 30 s [2].

Measurement of PKC activity

To measure PKC activity, approx. 100 mg of hepatocytes (10 mg dry wt./ml) were incubated under the same conditions as in the GS-DNP transport assay. After 10 min preincubation, the cells were treated either with 10 μM-staurosporine for 10 min or with 1 μg of PMA/ml for 3 min, followed by 10 μM-staurosporine for 10 min. The cells were then diluted with 1 volume of ice-cold phosphate-buffered saline (140 mM-NaCl, 9.2 mM-Na_2HPO_4, 1.3 mM-NaH_2PO_4) and centrifuged at 50 *g* for 3 min in a cooled centrifuge. The preparation was washed once with ice-cold phosphate-buffered saline. To assess viability of the cells, the ATP content and leakage of lactate dehydrogenase were determined in a sample of the cells. The cell pellets were resuspended in 2 ml of a buffer containing 2 mM-EGTA, 2 mM-EDTA, 50 μg of phenylmethanesulphonyl fluoride/ml, 0.01% leupeptin and 50 mM-Tris/HCl, pH 7.5 (buffer A), and sonicated with a Branson tip sonicator (3 × 3 s). The homogenate was centrifuged at 100000 *g* (Beckman Ultracentrifuge) for 60 min, yielding a soluble (cytosolic) and a particulate (pellet) fraction. To extract the membrane-bound enzyme, the pellet was resuspended by a short sonication (3 s) in 2 ml of buffer A supplemented with 1% Triton X-100, followed by a 15 min mixing period at 4 °C. Then the suspension was centrifuged at 100000 *g* for 20 min, yielding the solubilized membrane fraction.

The cytosolic and resolubilized fractions were partially purified by chromatography on a DEAE-cellulose (DEAE-Sephacel) column (volume 1 ml). The columns were equilibrated with 10 ml of buffer A (without leupeptin) supplemented with 1% Triton X-100; then 2 ml of the enzyme extract was added and the column was washed with 5 ml of the equilibration buffer, followed by 5 ml of equilibration buffer without Triton. The enzyme activity was eluted from the column by 2 ml of equilibration buffer (without Triton) supplemented with 150 mM-NaCl.

In experiments where staurosporine was used, Triton X-100 was omitted from the buffers used in the column step because of dissociation of staurosporine from PKC on the column in the presence of Triton.

The activity of PKC was determined using a kit from Amersham as described by the manufacturer. Briefly, samples were diluted 10-fold, and a 25 μl sample was incubated at 25 °C with 25 μl of a phospholipid/Ca^{2+} buffer containing a PKC specific peptide substrate and 25 μl of 150 μM-Mg^{2+}/ATP buffer containing 10 μCi of [^{32}P]ATP/ml. After 15 min the incubation was stopped and a 125 μl sample was spotted on filter paper. The filter papers were washed for 2 × 10 min in 5% (v/v) acetic acid with intermittent gentle mixing. Next, papers were counted for radioactivity in a liquid-scintillation counter. Blanks (incubations without the enzyme) were subtracted from the sample values. There was no significant Ca^{2+}/phospholipid-independent phosphorylation.

Activity was defined as pmol of phosphate incorporated/min per mg of protein in the sample.

Protein was determined as described by Bradford [14] with BSA as a standard.

Enzymic determinations

In all experiments, cell viability was measured by the release of lactate dehydrogenase activity from the cells into the medium and the cellular content of ATP. For determination of intracellular ATP, the cell fraction (bottom layer in 10% $HClO_4$ under silicone oil) was resuspended, centrifuged, and the protein-free supernatant of this fraction was neutralized with 3 M-potassium phosphate. ATP was determined fluorimetrically as described in [15]. Lactate dehydrogenase activity was measured as described in [16] in the medium fraction (supernatant of centrifugation through oil) and in the hepatocyte stock solution (for total activity).

RESULTS

Effect of PKC effectors on GS-DNP transport in normal and mutant hepatocytes

Hepatocytes from normal Wistar rats were preincubated at 37 °C with the phorbol ester PMA in concentrations ranging from 0 to 1000 ng/ml for 3 min (Fig. 1*a*), after which GS-DNP transport was measured. An increase in GS-DNP transport was

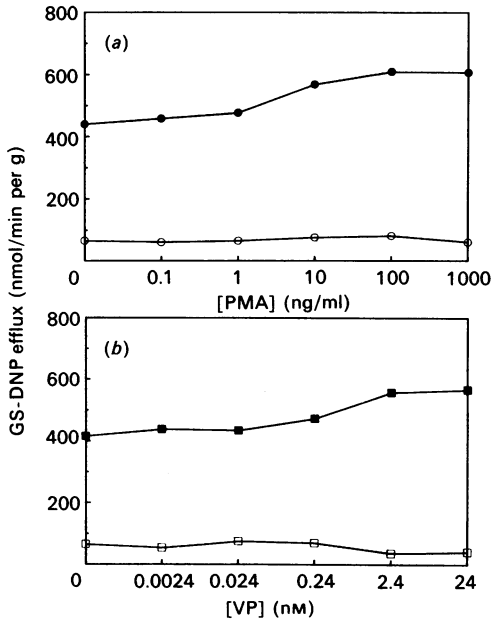

Fig. 1. Effect of PMA and VP on GS-DNP efflux from normal and mutant hepatocytes

Normal Wistar hepatocytes (●, ■) and mutant TR-hepatocytes (○, □) were preincubated for 3 min with the indicated concentrations of PMA (●, ○) or vasopressin (■, □). Subsequently, radioactively labelled CDNB was provided to the cells, and GS-DNP efflux was measured as described in the Materials and methods section. (*a*) Effect of PMA on GS-DNP efflux; (*b*) effect of VP on GS-DNP efflux. Results are means of two different experiments. Statistical data for a larger group of experiments with a single concentration of the stimulating agent are given in Table 3.

Table 1. Comparison of PKC levels and distribution of PKC in normal and mutant hepatocytes preincubated with or without PMA

Normal and mutant TR⁻ hepatocytes were incubated for 3 min with 1 μg of PMA/ml (stock is 1 mg/ml, dissolved in dimethyl sulphoxide), whereafter PKC activity was determined in the cytosolic and particulate fractions of cell homogenates, as described in the Materials and methods section. Control incubations were performed with dimethyl sulphoxide alone. Activity is expressed as pmol of phosphate incorporated/min per mg sample of protein. Values represent means ± S.D. of three separate experiments.

	Normal hepatocytes		TR⁻ hepatocytes	
	No PMA	+PMA	No PMA	+PMA
Total activity (pmol/min per mg)	3046±350	3114±396	5171±237	4853±84
% particulate	23±2.2	90±2.5	20±4.0	88±3.3

seen with PMA concentrations between 10 and 1000 ng/ml. Both in the presence and in the absence of PMA the secretion of GS-DNP was linear for periods up to 150 s.

To determine if the increase in GS-DNP secretion involved the canalicular transport system, we performed the same experiment with hepatocytes from the mutant TR⁻ rat, which has an inherited hepatocanalicular transport defect for a number of organic anions, including GS-DNP. As described previously, hepatocytes from TR⁻ rats secrete GS-DNP substantially slower than do Wistar rat hepatocytes [2]. TR⁻ hepatocytes preincubated with PMA did not show the increase in GS-DNP transport (Fig. 1*a*), indicating that the increase in GS-DNP transport seen with normal hepatocytes must be an increase in canalicular transport.

The latter conclusion is based partly on the assumption that mutant hepatocytes contain a normally functioning PKC system. This was assessed by measuring PKC activity and the distribution of PKC activity as a response to PMA in mutant TR⁻ cells. The results are compared with the situation in normal hepatocytes, as shown in Table 1. The results with mutant hepatocytes indicate that PKC activity is present; the activity is even substantially higher than in normal cells. The distribution pattern of PKC between cytosol and membrane in unstimulated cells, as well as PMA-treated cells, is similar to that in normal hepatocytes. These results indicate that PKC in mutant cells behaves in a similar way as in normal hepatocytes.

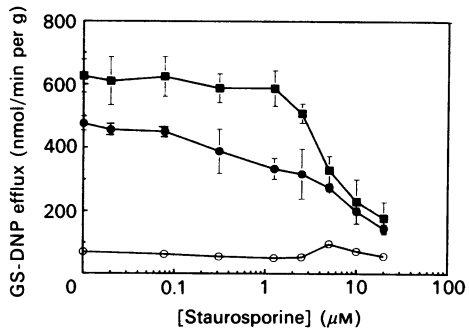

Fig. 2. Effect of staurosporine on GS-DNP efflux from normal unstimulated and PMA-stimulated hepatocytes and from unstimulated mutant hepatocytes

Normal hepatocytes were preincubated with staurosporine at the indicated concentrations for 10 min (●) or for 3 min with PMA (1 μg/ml) followed by 10 min incubation with staurosporine (■). Mutant hepatocytes were preincubated with staurosporine for 10 min (○). Subsequently, radioactively labelled CDNB was presented and GS-DNP efflux from the cells was measured as described in the Materials and methods section. Results are means of three different experiments ± S.D. for normal hepatocytes and means of two different experiments for the mutant TR⁻ hepatocytes. Statistical data for a larger group of experiments with normal hepatocytes with a single concentration of staurosporine are presented in Table 3.

Table 2. Effect of staurosporine on PKC activity of normal hepatocytes

Normal hepatocytes were incubated for 10 min with the indicated concentrations of staurosporine. PKC activity was measured in the cytosolic and particulate fractions as indicated in the Materials and methods section. PKC activity is expressed as the sum of the activities of the cytosolic and particulate fractions. Values represent means ± S.D. of three separate experiments.

[Staurosporine] (μM)	Total PKC activity (pmol/min per mg)
0	2487±66
0.1	1946±333
1	1469±405
10	678±165

GS-DNP transport was also measured in the presence of VP, a hormone known to stimulate the Ca^{2+}/PKC system in hepatocytes [17] (Fig. 1). The result is similar to that found with PMA; in normal hepatocytes a stimulation was seen with VP concentrations between 0.24 and 24 nM, whereas in mutant hepatocytes there was no stimulation of GS-DNP efflux.

Fig. 2 shows the result of preincubation of normal hepatocytes as well as mutant hepatocytes with the PKC inhibitor staurosporine (10 μM). Staurosporine inhibited GS-DNP efflux in normal cells, suggesting that these cells are already activated to some extent. Also, the increased GS-DNP efflux in cells stimulated by PMA could be decreased to the same level by staurosporine as in unstimulated cells. Staurosporine did not influence GS-DNP efflux in the mutant hepatocytes.

To verify that the decrease in GS-DNP efflux by staurosporine is due to an inhibition of PKC, we measured PKC activity in normal hepatocytes treated with staurosporine concentrations ranging from 0 to 10 μM (Table 2). Total cellular PKC activity was inhibited by staurosporine in the same concentration range as that inhibiting GS-DNP efflux (Fig. 2).

Table 3 shows a statistical analysis of the effects of PMA, VP and staurosporine on GS-DNP efflux from hepatocytes. VP

Table 3. Influence of modulators of PKC activity on GS-DNP efflux from rat hepatocytes

Normal Wistar-rat hepatocytes were preincubated for 3 min with PMA or VP or for 10 min with staurosporine. Subsequently GS-DNP efflux was measured as described in the Materials and methods section. The data were analysed by a paired Student *t* test of the given compound compared with the control incubation, and are presented as mean values ± S.D. for *n* experiments. For all compounds $P < 0.001$ compared with control.

Treatment	*n*	GS-DNP efflux (% of control)
Control		100
PMA (1 μg/ml)	11	155±28
VP (24 nM)	7	165±36
Staurosporine (10 μM)	8	47±13

Table 4. Effect of cAMP modulators on GS-DNP efflux from rat hepatocytes

Normal Wistar-rat hepatocytes were preincubated for 10 min with the indicated compounds. Subsequently GS-DNP efflux was measured as described in the Materials and methods section. The data are means ± S.D. from three separate experiments.

Treatment	GS-DNP efflux (% of control)
Control	100
Glucagon (100 nM)	98 ± 9
Forskolin (100 μM)	92 ± 15
db-cAMP (0.5 mM)	106 ± 8
IBMX (0.5 mM)	100 ± 11
Forskolin/IBMX (100 μM/0.5 mM)	100 ± 13

stimulated GS-DNP efflux to the same extent as PMA, compared with control efflux from cells without hormone treatment. Staurosporine (10 μM) inhibited efflux in unstimulated cells.

Effect of modulators of the cAMP system on GS-DNP efflux

We also tested the effect of modulators of the intracellular cAMP level on GS-DNP secretion. Table 4 shows that there is no significant effect of these modulators. Forskolin, a stimulator of adenylate cyclase, was not effective even in combination with the phosphodiesterase inhibitor IBMX. Also, the cAMP analogue db-cAMP and glucagon could not alter the GS-DNP efflux rate. All substances used, however, raised the glucose output of the hepatocytes by more than 50% compared with control incubations (results not shown), over an incubation period of 10 min, indicating activated glycogen breakdown.

DISCUSSION

In the present paper we show that organic-anion transport in isolated hepatocytes is stimulated by the hormone VP and the phorbol ester PMA, both activators of PKC. This transport was not stimulated in mutant TR^- hepatocytes, which have a defective canalicular organic-anion transport but normal sinusoidal efflux [2]. PKC is present in these mutant cells, and the PKC distribution between cytosol and membrane, under normal conditions as well as in the presence of PMA, is similar to the situation with normal hepatocytes. Taken together, this indicates that the stimulation of GS-DNP transport out of normal hepatocytes must be a stimulation of the canalicular transport, and thus of the MOAT system. In addition, staurosporine, an inhibitor of PKC, inhibits the GS-DNP efflux in both PMA-stimulated cells and unstimulated cells. Although staurosporine is not a specific inhibitor of PKC [18], the fact that the stimulation of GS-DNP transport by PMA, which is a specific activator of PKC [19], can be completely inhibited by staurosporine suggests that PKC is either directly or indirectly involved in regulation of the MOAT system. Furthermore, the staurosporine concentration range in which PKC activity was inhibited correlated well with the concentration that inhibited GS-DNP efflux, in both stimulated and unstimulated cells. The effective concentration of staurosporine used in our experiments is rather high compared with other reports [20–22], where concentrations between 0.1 and 1 μM were sufficient. These results, however, were found with other cell types than hepatocytes. The high concentration of staurosporine needed in hepatocytes may be due to a lower permeability of the hepatocyte membrane for staurosporine or to binding of staurosporine to cytosolic binding proteins such as ligandin, which would decrease the free intracellular concentration. Furthermore, there are indications [23] that staurosporine is a substrate for the MDR system, which is present at the canalicular side of hepatocytes [24,25]. Thus staurosporine might be pumped out of the cell by this system, thereby lowering the cytosolic steady-state concentration. This could explain the need for a higher extracellular staurosporine concentration to compensate for this effect.

The fact that staurosporine inhibits PKC activity as well as MOAT activity in cells which are not stimulated with PMA suggests that PKC in freshly isolated hepatocytes is partially activated, leading to a partially activated organic-anion transport. Indeed, we showed that 23% of the PKC activity is bound to membranes in the absence of added hormones. It has also been reported by others that in freshly isolated hepatocytes in the absence of stimulators already about 50% of PKC activity is found in the particulate fraction [26]. This could be due to circulating hormones, induced during surgical procedures for isolation of hepatocytes. On the other hand, it is possible that, *in vivo* under basal conditions, PKC in hepatocytes is already partially activated in hepatocytes, and that further activation can be achieved by hormones in the bloodstream.

The mechanism of regulation of canalicular secretion is not yet clear. Most likely there is a direct phosphorylation by PKC of the MOAT, but it is also possible that the intracellular concentration of GS-DNP is changed, owing to PKC activation. It is known that glutathione conjugates of nitrophenyl compounds can bind to glutathione *S*-transferases (GST) in the cytosol [27]; this means that the free concentration of GS-DNP in the cytosol is determined by the affinity for these binding proteins. Taniguchi *et al.* [28] reported that some of the GST isoenzymes are good substrates for PKC *in vitro* and they found that isoenyzme 1-1 (ligandin) has a 2-fold lower affinity for the organic anion bilirubin upon phosphorylation. By analogy, this means that the intracellular free GS-DNP concentration could increase in response to PKC activation, giving rise to an increased transport rate. A prerequisite for this possibility is that a substantial part of GS-DNP is bound to GST. In our transport assay we use a concentration of 30 μM-CDNB with a cell density of 10 mg dry wt./ml, and the intracellular volume is 2.7 ml/g dry wt. [2]. One can calculate that after uptake and conjugation of all the CDNB, the internal GS-DNP concentration will be approx. 1.1 mM. (2-Chloro-4-nitrophenyl)-*S*-glutathione, a compound that resembles GS-DNP, was found to have a dissociation constant (K_d) of 0.34 μM [27]. Taken together with a cytosolic GST concentration of 0.2 mM (assuming that 10% of the cytosolic protein is GST [29] and the K_d of ligandin for GS-DNP is similar), one can deduce that the GST pool will be virtually saturated by GS-DNP at the concentration we used. Thus it is not likely that a change of GST affinity contributes to a major extent to the stimulation of GS-DNP efflux from the hepatocytes. Furthermore, if the increase in GS-DNP transport is caused by a change in the intracellular free GS-DNP concentration, one would expect that the residual transport in TR^- hepatocytes, which is probably sinusoidal transport and is not saturated under the conditions used in the present experiments [2], would also increase upon PMA treatment. This is not the case. Also, the GST content of TR^- hepatocytes is normal [2]. This leaves a direct phosphorylation of the MOAT as the most probable explanation for the observed effect.

The PKC-mediated stimulation of GS-DNP transport via MOAT constitutes an additional resemblance of this transporter to members of a superfamily of ATP-dependent transport systems [30], including P-glycoprotein (associated with MDR) and the cystic fibrosis gene product [31–34]. Both P-glycoprotein and

the cystic fibrosis gene product have phosphorylation sites [35]. Furthermore, P-glycoprotein was recently shown to be phosphorylated by PKC, causing a stimulation of transport [11,12].

The role of a regulatory mechanism, as described in the present paper, in bile formation is not clear. Bile formation is a complex process in which many factors are involved (for reviews see [36–38]). Bile flow is determined by an osmotic gradient between blood and bile. This is the driving force for water and small molecules, which enter bile through the tight junctions between hepatocytes. The osmotic pressure is established, for a large part, by active transport of bile acids (bile-acid-dependent fraction). For the remaining part (bile-acid-independent fraction) bicarbonate and possibly other organic anions, such as glutathione, are thought to be responsible. The latter compound is transported by MOAT [2,39], and this transport can be regulated by PKC, as shown in the present paper. On the basis of these findings, one would expect a small stimulation of bile flow by PKC activators, such as phorbol esters. However, Corasanti *et al.* [40] have shown, in the perfused rat liver, that phorbol ester inhibits bile secretion independently of effects on the microcirculation. One explanation might be that in the intact liver Kupffer cells, endothelial cells and bile-duct cells may also be stimulated by phorbol ester. This could have an effect on bile formation, through the formation of mediators such as prostaglandins, leukotrienes, platelet-activating factor (PAF) or cytokines. In addition, VP and adrenaline, both activators of the Ca^{2+}/PKC second-messenger system, have been reported to increase paracellular permeability, thus allowing a dissipation of the osmotic gradient. This may result in a decrease in bile flow, but this effect is thought to be caused by the Ca^{2+} component of the signal-transduction pathway, rather than PKC [41]. In a recent abstract, Nathanson & Boyer [42] reported that phorbol ester decreases the canalicular volume of rat hepatocyte couplets without affecting tight-junctional permeability. Translated to the whole organ, this may indicate an inhibition of bile formation. Although the conditions in which these experiments were performed differ from the situation *in vivo*, one can conclude that PKC probably is also coupled to other factors involved in bile formation apart from organic-anion transport. Thus the physiological importance of the regulation of organic-anion transport described in the present paper remains uncertain.

In conclusion, the results presented here show that canalicular transport of glutathione conjugates is regulated via PKC. Whether this is caused by a direct phosphorylation of the glutathione-conjugate transporter, MOAT, remains a subject for further investigation.

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