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Essential Role of Sodium and Chloride for Theophylline-Induced Choleresis in the Isolated Perfused Rat Liver (41844)

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Abstract. Active secretion of electrolytes by hepatocytes is believed to be responsible for bile acid-independent canalicular bile flow (BAICF). Theophylline, which enhances BAICF, has been shown to enhance electrogenic Cl^- secretion in a number of other epithelia. Such transport is dependent on Na^+ and Cl^-. Thus, the mechanism of theophylline choleresis may also involve stimulation of electrogenic Cl^- secretion of the liver. This hypothesis was tested by studying the effect of ion substitution on theophylline choleresis in isolated perfused rat livers. Addition of theophylline (0.1 mmole) and dibutyryl cAMP (0.05 mmole) to 100 ml perfusate, as a single dose, increased bile flow and biliary secretion of Na^+ and Cl^- reversibly. These effects of theophylline were virtually abolished when perfusate Na^+ (146 m*M*) was replaced by Li^+ (146 m*M*) or choline$^+$ (120 m*M*), and when Cl^- (127 m*M*) was replaced by 120 m*M* NO_3^-, acetate$^-$ or isethionate$^-$. Since even the permeable ions like Li^+ and NO_3^- could not substitute for Na^+ and Cl^-, these results show that the effect of theophylline on BAICF is specifically dependent on the presence of Na^+ and Cl^- in the perfusate. We propose, by analogy to other epithelia, that an electrogenic Cl^- secretion mechanism is present in the liver. Theophylline, acting via cAMP, stimulates this transport process, thereby enhancing BAICF.

A fraction of canalicular bile flow is independent of hepatic bile acid secretion and is stimulated by a number of drugs without changing the rate of bile acid secretion (1). This fraction is commonly known as the bile acid-independent canalicular bile flow (BAICF). Existing evidence suggests that active transport of inorganic electrolytes into the canaliculi is responsible for the BAICF (1–3). However, the mechanism of hepatic electrolyte transport and its relation to bile formation are not clearly understood. The major obstacle to the clarification of hepatic electrolyte transport is our inability to sample bile and conduct electrophysiological studies at the level of canaliculus. Nonetheless, the availability of pharmacological agents, which selectively alter one or more transport functions in other epithelia, should permit further insight into the mechanism of hepatic electrolyte and fluid transport.

Theophylline is a drug known to stimulate the BAICF of dogs (4, 5) and rats (6) *in vivo*. The mechanism of theophylline-induced choleresis is considered to be via cAMP, since theophylline is a potent inhibitor of phosphodiesterase. In unrelated studies using other epithelia, it has been shown that procedures which result in an elevated intracellular cAMP and/or cytosolic free Ca^{2+} level, also enhance electrogenic Cl^- secretion (7). Together, these experimental findings raise the possibility that electrogenic Cl^- secretion is involved in BAICF. Studies using other epithelia further showed that electrogenic Cl^- secretion is dependent on both Na^+ and Cl^- (7). If such a mechanism exists in liver, and if theophylline acts similarly as in other epithelia, then theophylline should stimulate BAICF by enhancing Cl^- transport across hepatocytes. If this condition is satisfied, the choleretic effect of theophylline should be associated with an increase in the biliary secretion of Na^+ and Cl^-, and should be dependent on both Na^+ and Cl^-.

In the present study, we examined the effect of theophylline on biliary secretion of Na^+

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and Cl^- using isolated perfused rat livers. Ion-substitution studies were performed to test whether Na^+ and Cl^- are required to exhibit the choleretic effect of theophylline.

Materials and Methods. *Materials.* Theophylline, lactate dehydrogenase, 3-hydroxysteroid dehydrogenase, NAD, and NADH were obtained from Sigma Chemical Company, Munich, West Germany. Taurocholate (TC) was purchased from Calbiochem, San Diego, California. [^{14}C]Erythritol was purchased from New England Nuclear, Boston, Massachusetts. All other chemicals were products of Merck, Darmstadt, West Germany. Inbred male Wistar rats (250–350 g) maintained on a standard laboratory diet (Altromin) served as liver donors in all experiments. Liver weights varied from 7 to 13 g.

Isolated perfused rat liver. Each liver was surgically prepared by methods previously described (8), connected to the perfusion apparatus, and maintained at 37°C (9). They were perfused with 100 ml of recirculating erythrocyte-free perfusate (pH 7.4) under a hydrostatic pressure of 15 cm. Erythrocyte-free perfusion medium was used to avoid any possible effect of ion replacement on erythrocytes. The perfusion rate through the liver was initially adjusted to 4–5 ml/min × g liver to ensure an adequate oxygen supply (10). The pH was maintained between 7.3 and 7.4 by addition of 0.15 *M* HCl, $NaHCO_3$, LiOH, HNO_3, or acetic acid, dependent upon the perfusate medium. The routine viability criteria included: (i) stable perfusion rate throughout the experiment, (ii) perfusate lactate/pyruvate ratio around 10, and (iii) relatively stable bile flow during the control period. Experiments which did not satisfy these criteria were not included.

The composition of the basic perfusion medium was as follows: 120 m*M* NaCl, 4.7 m*M* KCl, 26 m*M* $NaHCO_3$, 1.2 m*M* KH_2PO_4, 1.3 m*M* $CaCl_2$, 0.6 m*M* $MgSO_4$, and 5.5 m*M* glucose. In order to study the effect of Na^+ and Cl^- on bile formation, NaCl was replaced by an equimolar concentration of choline-Cl, $NaNO_3$, Na-acetate, or Na-isethionate. Complete replacement of Na^+ and Li^+ was obtained by substituting NaCl and $NaHCO_3$ with equimolar concentrations of LiCl and $LiHCO_3$, respectively. The perfusates were gassed with 95% O_2 and 5% CO_2. Viability criteria were not affected significantly by these ion substitutions (8).

Experimental design. The liver was perfused with the appropriate experimental perfusate from the start of each experiment. Theophylline (0.1 mmole; perfusion concentration 1 m*M*) was injected as a single dose after 60 min of perfusion. Bile was collected in a preweighed vial and the bile volume was determined gravimetrically assuming a density of 1.0. Perfusate samples were collected at the midpoints of the bile collection periods. Erythritol clearance was studied routinely by adding 2 μCi of [^{14}C]erythritol to the reservoir at the start of each experiment. The bile to plasma ratio of [^{14}C]erythritol was close to 1 in each experiment (1.06 ± 0.054; mean ± SD), indicating canalicular origin of bile.

Isolated plasma membranes from rat liver. Isolated plasma membranes were used to determine if theophylline affects the activity of sodium and potassium activated Mg^{2+}-ATPase (Na^+,K^+-ATPase). Plasma membranes were isolated under isotonic conditions according to the method of Coleman *et al.* (11). The purity of the plasma membranes was routinely followed by measuring the activities of marker enzymes for plasma membranes (5′-nucleotidase and Na^+, K^+-ATPase), endoplasmic reticulum (glucose-6-phosphatase) and mitochondria (succinate dehydrogenase). Specific activities of our marker enzymes have been previously published (8). Protein content of plasma membranes was determined by the method of Lowry *et al.* (12) using bovine serum albumin as standard. The activity of Na^+,K^+-ATPase was determined by the method of Emmelot *et al.* (13).

Analytical methods. The concentrations of Na^+, K^+, and Li^+ in perfusate and bile were determined using flame photometry. Chloride concentrations were determined by coulometric titration using a chloridometer (Corning, Model 920). Radioactivity was measured in a liquid scintillation counter with quench corrected by external standard ratios. Total bile acids were determined by an enzymatic method using 3-hydroxysteroid dehydrogenase (14). The perfusate lactate and pyruvate concentrations were determined enzymatically using lactate dehydrogenase. Osmolarity was

measured by the freezing-point depression method using a microosmometer (Kanuer, West Germany). Bile was isosmotic to perfusate in all studies.

Statistical methods. The effect of drug on bile flow and biliary secretion of Na^+ and Cl^- was determined by comparing values after injection of drug at 60 min to the average value of 30- to 60-min collection periods (predrug controls). The paired *t* test was used to detect differences in experimental parameters with $P < 0.05$ considered significant.

Results. *1. Effect of sodium replacement on theophylline choleresis.* Preliminary studies showed that addition of 0.1 mmole theophylline to 100 ml perfusate produced consistent choleresis. Thus, a 0.1 mmole dose of theophylline was used in all studies. Addition of theophylline after 60 min of perfusion with a Na^+-containing perfusate resulted in a rapid increase in bile flow (Fig. 1) which remained significantly elevated through 120 min. When perfusate Na^+ was replaced by Li^+, theophylline caused an initial decline in bile flow followed by an increase with a peak value at 80 min, then a second decline back to basal level at 90 min (Fig. 1). Theophylline caused a slight increase in bile flow when perfusate Na^+ (145 m*M*) was partially replaced by choline$^+$ (120 m*M*) (Fig. 1). Complete replacement of Na^+ by choline$^+$ was not studied since this results in erratic perfusion flow (10). Theophylline did not significantly affect the viability of the perfused liver under our experimental conditions (Table I). These results indicate that neither Li^+ nor choline$^+$ can completely replace Na^+ in theophylline choleresis.

The biliary concentrations of Na^+ and Cl^- were similar to their perfusate concentrations and were not significantly affected by theophylline. Thus, the biliary secretion of these electrolytes paralleled bile flow. Theophylline increased biliary secretion of Na^+ and Cl^- when livers were perfused with NaCl-containing perfusate. Replacement of Na^+ by Li^+ or choline$^+$ reduced the theophylline-induced increase in biliary Cl^- secretion (Fig. 1). The low endogenous bile acid secretion was not affected by theophylline (1.61 ± 0.42 vs 1.48 ± 0.37 nmole/min × g liver, $N = 30$). These results indicate that theophylline-induced bile

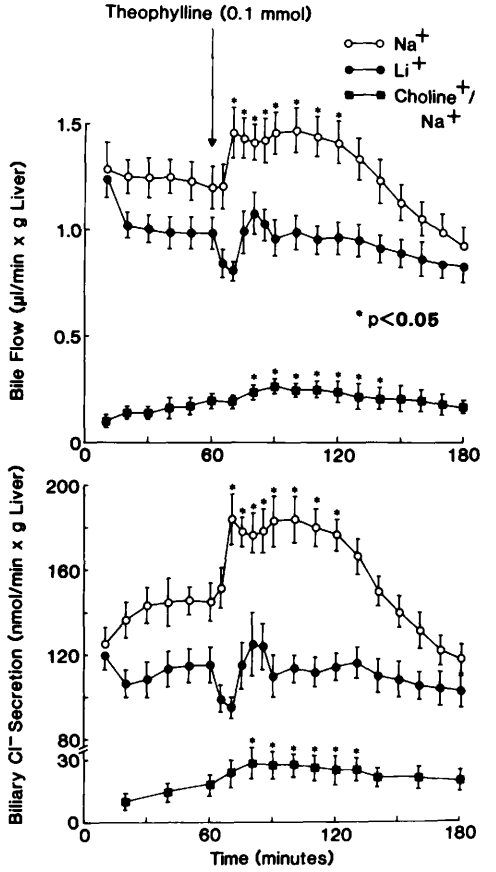

FIG. 1. Effect of Na^+ replacement on theophylline-induced bile flow and biliary Cl^- secretion. *Significantly higher than the 30- to 60-min control values. Means ± SE ($N = 5$).

flow and biliary Cl^- secretion is Na^+ dependent.

To determine if this Na^+ dependency is due to activation of Na^+,K^+-ATPase, the effect of theophylline on the activity of this enzyme in plasma membranes from isolated rat livers was determined. Theophylline up to a concentration of 10 m*M* failed to activate this enzyme (Table II). Thus, it seems unlikely that the effect of theophylline is directly mediated via Na^+,K^+-ATPase.

2. Effect of chloride replacement. Theophylline produced very little, if any, choleresis when the perfusate Cl^- (127 m*M*) was replaced by 120 m*M* NO_3^-, acetate$^-$, or isethionate$^-$. The biliary concentration of Cl^- under these

TABLE I. EFFECT OF THEOPHYLLINE AND DB cAMP ON VIABILITY OF ISOLATED PERFUSED RAT LIVERS[a]

Perfusate	Treatment	Perfusate flow (ml/min × g liver)		Lactate/pyruvate ratio		Perfusate K^+ concentration (mmole/ liter)	
		1st hr	2nd hr	1st hr	2nd hr	1st hr	2nd hr
NaCl + $NaHCO_3$	Control	4.7 ± 0.13	4.8 ± 0.12	11 ± 1.8	12 ± 2.1	5.6 ± 0.14	5.8 ± 0.16
	Theophylline	4.6 ± 0.11	4.6 ± 0.13	10 ± 1.3	12 ± 1.8	5.6 ± 0.15	5.7 ± 0.13
	DB cAMP	4.5 ± 0.12	4.6 ± 0.11	11 ± 1.4	11 ± 1.3	5.5 ± 0.12	5.8 ± 0.14
Choline-Cl + $NaHCO_3$	Control	4.7 ± 0.14	4.8 ± 0.13	10 ± 1.8	11 ± 1.7	5.9 ± 0.26	7.8 ± 0.48
	Theophylline	4.8 ± 0.15	4.7 ± 0.16	9 ± 1.6	11 ± 1.4	6.0 ± 0.23	7.7 ± 0.39
LiCl + $LiHCO_3$	Control	4.5 ± 0.13	4.6 ± 0.15	12 ± 1.7	12 ± 1.8	6.6 ± 0.34	8.4 ± 0.56
	Theophylline	4.5 ± 0.14	4.5 ± 0.17	10 ± 1.3	11 ± 1.4	6.5 ± 0.28	8.6 ± 0.61
$NaNO_3$ + $NaHCO_3$	Control	4.9 ± 0.13	4.8 ± 0.15	11 ± 1.4	10 ± 1.1	5.7 ± 0.18	5.9 ± 0.21
	Theophylline	4.6 ± 0.12	4.6 ± 0.13	12 ± 1.3	11 ± 1.4	5.6 ± 0.13	5.8 ± 0.19
Na-acetate + $NaHCO_3$	Control	4.5 ± 0.15	4.6 ± 0.17	11 ± 1.6	11 ± 1.5	5.7 ± 0.19	5.7 ± 0.15
	Theophylline	4.9 ± 0.17	4.8 ± 0.16	12 ± 1.3	13 ± 1.6	5.6 ± 0.20	5.9 ± 0.17
Na-isethionate + $NaHCO_3$	Control	4.7 ± 0.16	4.7 ± 0.20	10 ± 1.8	9 ± 0.7	5.8 ± 0.16	5.9 ± 0.20
	Theophylline	4.5 ± 0.18	4.6 ± 0.21	11 ± 1.3	11 ± 1.4	5.5 ± 0.13	5.7 ± 0.16

Note. Values are means ± SEM of 30 determinations (six determination for each hour and experiment).
[a] Theophylline also did not significantly affect these parameters during the 3rd hour of perfusion.

conditions was less than 10 m*M* and was not altered by theophylline. The theophylline-induced increase in biliary Na^+ secretion was also virtually abolished by Cl^- replacement (Fig. 2). The viability of the perfused liver was not affected significantly by theophylline under these conditions (Table I). Since even the permeable NO_3^- could not substitute for Cl^-, these results indicate that Cl^- is specifically required for theophylline-induced Na^+ secretion and bile flow.

3. Effect of dibutyryl cAMP (DB cAMP). If theophylline acts via cAMP, DB cAMP should also increase bile flow and biliary electrolyte secretion. Addition of 50 μmole of DB cAMP promptly increased bile flow and biliary secretion of Na^+ and Cl^- (Fig. 3). DB cAMP did not significantly affect the viability of the perfused liver (Table I) nor the biliary bile acid secretion (1.82 ± 0.52 vs 1.76 ± 0.42 nmole/min × g liver, $N = 30$).

TABLE II. EFFECT OF THEOPHYLLINE ON RAT LIVER PLASMA MEMBRANE Na^+, K^+-ATPase ACTIVITY

Theophylline concentration (m*M*)	Na^+,K^+-ATPase activity (μmole P_i/hr × mg protein)
0	14.0 ± 0.87
0.01	13.7 ± 0.90
0.1	13.8 ± 0.89
1.0	13.7 ± 0.91
10.0	13.9 ± 0.88

Note. Values are means ± SEM ($N = 8$).

Discussion. These studies were designed to determine if the choleretic effect of theophylline could be explained by the proposed mechanism of action of theophylline in other epithelia (7). Our results clearly indicate that theophylline stimulates canalicular bile formation in the isolated perfused rat liver. This choleresis is associated with an increase in biliary secretion of both Na^+ and Cl^-. Since endogenous bile acid secretion was not altered, this effect cannot be due to theophylline-induced secretion of bile acid.

Replacement of Na^+ and Cl^- by other ions virtually abolished the effect of theophylline on bile flow and biliary secretion of Na^+ and Cl^-. Since the viability of the perfused liver was not significantly affected (Table I), the failure of theophylline to increase bile flow and biliary electrolyte secretion when Na^+ and

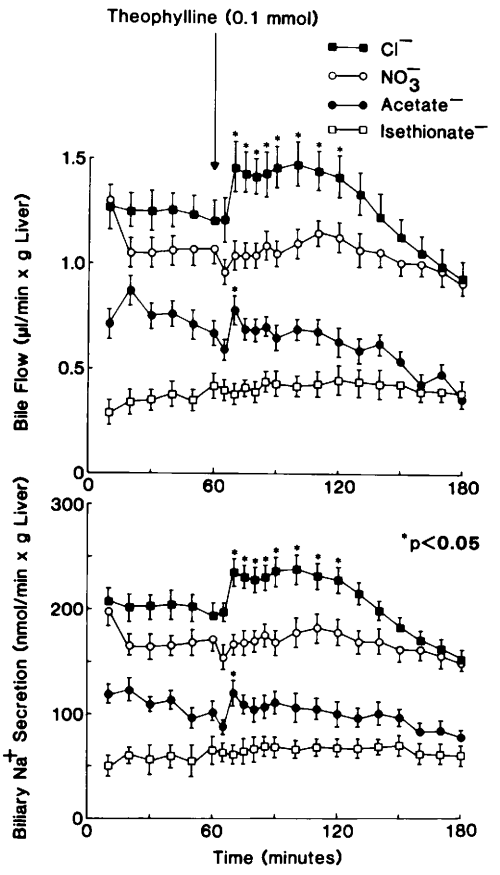

FIG. 2. Effect of Cl^- replacement on theophylline induced bile flow and biliary Na^+ secretion. *Significantly higher than the 30- to 60-min control values. Means ± SE (N = 5).

Cl^- were replaced by other ions is unlikely to be due to any toxic effect of theophylline. Thus, the effect of theophylline is dependent on both Na^+ and Cl^-. Since even the permeable ions, such as Li^+ and NO_3^-, could not substitute for Na^+ and Cl^-, it is unlikely that a passive mechanism is responsible for theophylline-induced biliary secretion of these ions. We previously showed that the choleretic effect of taurocholate, an osmotic choleretic, increased when Na^+ and Cl^- were replaced by Li^+ and NO_3^- (15). A similar effect would have been anticipated if theophylline had been acting as an osmotic choleretic. Since the choleretic effect of theophylline declined under these conditions, it is unlikely that the choleretic effect of theophylline is due to the osmotic effect of the secreted drug. Moreover, studies in dogs (16) indicated that the choleretic effect of theophylline cannot be explained by the osmotic effect of the secreted drug alone. Thus, it is more likely that there is a specific transport system for these ions and that theophylline acts by modifying this transport system. Possible modifications of Na^+,K^+-ATPase seem unlikely since theophylline up to a concentration of 10 mM does not affect the activity of this enzyme in liver plasma membranes (Table II).

The effects of theophylline observed in these studies are similar to effects in other epithelia (7). Thus, it is likely that theophylline acts via similar mechanisms in the liver. Theophylline

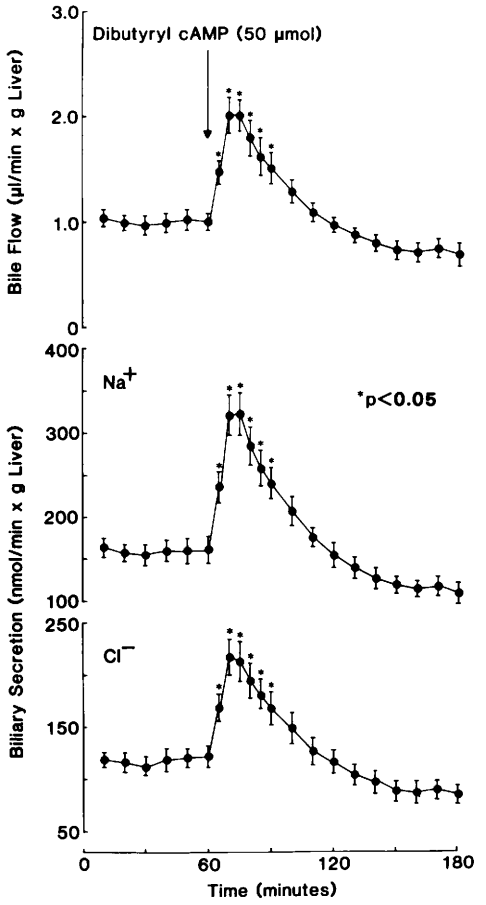

FIG. 3. Effect of dibutyryl cAMP on bile flow and biliary secretion of Na^+ and Cl^-. *Significantly higher than the 30- to 60-min control values. Means ± SE (N = 5).

is known to stimulate electrogenic Cl^- secretion and inhibit Na^+-coupled Cl^- reabsorption in different epithelia (7). Both processes are dependent on Na^+ and Cl^- (7). Since theophylline-induced effects in our studies are also dependent on both Na^+ and Cl^-, either or both mechanisms may be involved in the liver. Unfortunately, our studies cannot distinguish between these two possibilities. However, since there is no compelling evidence that indicates theophylline induces reabsorption of fluid from canaliculi, we propose that theophylline increases BAICF by stimulating electrogenic Cl^- secretion. In this case, the electrogenic Cl^- transport would involve secretion of Cl^- across the canalicular membrane followed by Na^+ through the paracellular pathway in order to maintain electroneutrality.

Frizzel *et al.* (7) proposed that electrogenic Cl^- secretion may involve Na^+-coupled Cl^- transport at the contraluminal membrane, i.e., sinusoidal membrane of liver cells. The present findings indicate that a major fraction of theophylline-induced Cl^- secretion is specifically dependent on Na^+ (Fig. 1) which support the hypothesis of Frizzel *et al.* A similar mechanism has been proposed in recent reviews (3, 13), and was supported by our previous ion-replacement studies in isolated perfused rat livers (8). However, attempts to obtain direct evidence for the presence of Na^+-coupled Cl^- transport were unsuccessful in cultured rat hepatocytes (17). Thus, biliary Cl^- secretion may not involve Na^+-coupled Cl^- transport at the sinusoidal membrane. Alternatively, this transport mechanism may be present in the liver, and may become quantitatively important only during theophylline choleresis.

The present study showed that although replacement of Na^+ by Li^+, and Cl^- by NO_3^- decreased basal bile flow only slightly, the choleretic effect of theophylline was virtually abolished in the presence of Li^+ and NO_3^-. These differences in the effect of Li^+ and NO_3^- indicate that more than one ion transport mechanism is involved in the formation of bile acid independent bile. One of those mechanisms seems to be specific for Na^+ and Cl^-, and is stimulated by theophylline.

This study also showed that DB cAMP stimulates bile flow and biliary secretions of Na^+ and Cl^- (Fig. 3). Thomsen and Larsen (18) also reported that DB cAMP is choleretic in rats and in the isolated perfused rat liver. Since theophylline is known to act via cAMP, these results are in agreement with the hypothesis that theophylline acting via cAMP stimulates BAICF. This mechanism has been questioned by others. Baker and Kaplan (19) reported that intraperitoneal injection of DB cAMP failed to stimulate bile flow in rats, and Poupon *et al.* (20) reported that an increase in hepatic cAMP levels induced by various drugs was not always associated with choleresis in rats. Since Baker and Kaplan (19) injected DB cAMP intraperitoneally, it is possible that an effective hepatocellular concentration was not achieved. The results (20) indicating that increases in cAMP levels were not always associated with choleresis are difficult to explain. It is, however, also possible that drugs used to increase hepatic cAMP levels may have other effects which in turn might have influenced bile flow. Other possibilities may include compartmentation of cAMP in liver cells. Since theophylline and DB cAMP are also choleretic in dog and man (4, 5, 16, 21), it seems probable that cAMP plays a role in BAICF and that theophylline acts via cAMP in liver cells.

In conclusion, the present study provides indirect evidence for the presence of an electrogenic Cl^- secretory mechanism in liver cells. Theophylline acting via cAMP, stimulates this transport system thereby enhancing bile acid-independent canalicular flow.

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