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Endocrine and Paracrine Calcium Signaling in Bile Duct Cells

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Bile duct cells play an important role in maintaining, modifying and augmenting bile flow. It is well established that cyclic AMP (cAMP) is an important second messenger for secretion in these cells, but less is known about cytosolic Ca^{2+} (Ca_i^{2+}). Here we review evidence that ATP and acetylcholine (ACh) are Ca_i^{2+} agonists for bile duct cells, and that these agonists increase Ca_i^{2+} through inositol 1,4,5-trisphosphate ($InsP_3$). We also review data suggesting that hepatocytes have the ability to secrete ATP, so that they may serve as a paracrine source for this signaling molecule *in vivo*. Finally, we compare the effects of cAMP and Ca_i^{2+} on secretion, both in isolated bile duct units and isolated hepatocyte couplets. Implications and future directions for studying the role of Ca_i^{2+} in bile ductular secretion are discussed.

INTRODUCTION

Bile duct epithelial cells, or cholangiocytes, serve as the inner lining for the biliary tree. It was long thought that these cells merely served as a conduit for bile secreted by hepatocytes as that bile moved through and out of the liver. Now, though, there is considerable evidence that bile duct cells also significantly modify and condition the primary secretions of the hepatocyte canaliculus [1, 2]. For example, bile duct cells can secrete additional bile, and secretin is one hormone that induces this [3, 4]. Secretin's actions are mediated by cyclic AMP (cAMP)[b] [3], which in turn stimulates exocytosis [5], activates the CFTR Cl^- channel [6] and inserts additional water channels into the plasma membrane [7]. Cytosolic Ca^{2+} (Ca_i^{2+}) modulates secretion in other epithelial cells [8], including hepatocytes [9], and there is electrophysiological evidence that cholangiocyte plasma membranes contain Ca^{2+}-activated Cl^- channels [10, 11]. Therefore, it may be hypothesized that agents that increase Ca_i^{2+} in cholangiocytes would act as cholangiocyte secretagogues as well. Here we review studies to determine which agonists increase Ca_i^{2+} in bile duct cells and discuss both the mechanisms by which these agonists increase Ca_i^{2+} and the effects of Ca_i^{2+} signaling on secretion in these cells.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats (180-250 g) were used for all experiments. Adenosine triphosphate (ATP), uridine triphosphate (UTP), acetylcholine (ACh), thapsigargin, grade III apyrase, [Arg8]vasopressin, forskolin, 1-9-dideoxyforskolin and propidium iodide were obtained from Sigma (St. Louis, MO); fluo-3/AM and indo-1/AM were obtained

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[b] *Abbreviations:* cAMP, cyclic AMP; $InsP_3$, inositol 1,4,5-trisphosphate; ACh, acetylcholine; CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-1-methylxanthine.

from Molecular Probes (Pitchford, OR); and suramin was obtained from Biomol (Plymouth Meeting, PA). All other chemicals were of the highest quality commercially available.

Preparation of isolated hepatocytes, hepatocyte couplets and bile duct units

Isolated rat hepatocytes, bile duct cells and bile duct units were prepared in the Cell Isolation Core of the Yale Liver Center as described previously [12-15]. Briefly, to isolate hepatocytes, rat livers were perfused with collagenase-containing medium, then excised, minced and passed through serial nylon mesh filters, and the resultant cells were washed. These cells were suspended in Leibovitz L-15 medium containing 10 percent fetal calf serum, 50 U penicillin and 50 mg streptomycin/ml and plated onto glass coverslips. Cells were incubated at 37°C and used 2-6 hr after plating. Viability of hepatocytes by trypan blue exclusion was measured 2 hr after plating and exceeded 90 percent. To obtain isolated bile duct units, livers were perfused with collagenase-containing medium as described above, then the portal tissue residue was mechanically separated from parenchymal tissue. The tissue was then minced and sequentially filtered. Fragments remaining on the filters were digested and filtered further, then plated on coverslips coated with Matrigel. This isolation procedure results in aggregates of bile duct cells that are 30-100 μm in diameter. These bile duct cells were used for studies ~24 hr after plating. In selected experiments, the medium was replaced with L-15 medium after 20-24 hr, then hepatocytes isolated as described above were plated on the same coverslips for 2-4 hr, in order to examine co-cultures of hepatocytes and bile duct cells [16].

Microinjection experiments

Micropipettes were used for mechanical stimulation and microinjection. The micropipettes were commercially obtained from Eppendorf (Madison, WI) and had an internal diameter of less than 0.5 μm. A series 5171 Eppendorf micromanipulator was used for positioning, and an Eppendorf series 5242 microinjector was used for pressure microinjections. For injections, micropipettes were loaded with 150 mM KCl, 1 mM HEPES, either heparin (1 mg/ml) or de-N-sulfated heparin (1 mg/ml), plus Texas red (0.4 mg/ml) as a marker of injection [13].

Confocal microscopic measurements of cytosolic Ca^{2+}

Rat hepatocytes and bile duct units were isolated and plated onto glass coverslips as described above, then loaded with the Ca_i^{2+}-sensitive fluorescent dye fluo3/AM (6 μM). The coverslips were transferred to a chamber on the stage of a Zeiss Axiovert microscope, then the cells were perfused at 37°C and observed using a BioRad MRC-600 confocal imaging system. A krypton-argon laser was used to excite the dye at 488 nm, and emission signals above 515 nm were collected [8, 13]. Cells were stimulated under various conditions and the resulting Ca_i^{2+} signals were video-recorded at a rate of 1 frame/sec [13, 16]. In selected experiments, Ca_i^{2+} signals were instead detected using confocal line scanning microscopy [8].

In other experiments, the fluorescent nuclear stain propidium iodide (10 μM) was present in the perifusion medium to identify disrupted or dead cells [16]. In these studies, cells were excited with both the 488 nm and 568 nm excitation lines of the krypton-argon laser. Confocal images were recorded at two separate emission wavelengths: the first one centered at 522 nm and the second one greater than 585 nm, to detect fluorescence from fluo-3 and propidium iodide, respectively. This approach permitted Ca_i^{2+} signaling (i.e., fluo-3 fluorescence) and cell membrane integrity (i.e., propidium iodide uptake) to be monitored simultaneously [16].

Measurement of secretion in bile duct units and hepatocyte couplets

Secretion was determined in isolated bile duct units and hepatocyte couplets by serial measurements of their luminal volumes over time. These volumes were determined using optical planimetry, by measuring the cross-sectional area of serial optical sections through the luminal space [17, 18]. Rat hepatocyte couplets or bile duct units were isolated as described above, then transferred to a temperature-controlled perifusion chamber on the stage of a Zeiss IM35 inverted microscope, and cells were observed through a 63x objective lens. Nomarski optics were used to obtain a shallow (0.25-0.3 μm) depth of focus, and the cells were imaged with a Dage-MTI Series 68 video camera [12, 17, 18]. Images were recorded on an optical disc, and luminal areas were determined from the recorded images using an image processor.

Statistics and data analysis

Given the wide variability in responses that is seen among cells in primary culture [19], treatment groups were compared only to those control groups isolated from the same liver preparations. Values listed are mean ± SD. Statistical comparisons were made using student's t test, and p-values less than .05 were taken as significant.

RESULTS AND DISCUSSION

Identification of Ca^{2+} agonists for bile duct cells

The effects of a range of potential agonists on Ca_i^{2+} were examined, using both ratio microspectrofluorometry and confocal microscopy [13]. Vasopressin (10 nM), angiotensin II (10 nM) and phenylephrine (10 μM) each cause an inositol 1,4,5-trisphosphate ($InsP_3$)-mediated increase in Ca_i^{2+} in hepatocytes [8, 12], but none of these agents increased Ca_i^{2+} in bile duct units (n = 12 experiments for each agonist). Similarly, cholecystokinin (500 pM) and bombesin (10-100 nM) each increase Ca_i^{2+} in pancreatic epithelia [8, 20], but neither of these agents increased bile duct cell Ca_i^{2+} (n = 10 and 11 experiments, respectively). However, acetylcholine (ACh, 10 μM-1 mM) is another Ca_i^{2+} agonist for exocrine pancreas [8, 20] and similarly increased Ca_i^{2+} in bile duct cells (n > 100 observations) [13]. This effect of ACh was blocked by atropine (1 μM). ACh had no effect on Ca_i^{2+} in isolated hepatocytes, however (n = 8 experiments; 14 ± 1 hepatocytes/experiment). In addition, both ATP and UTP increase Ca_i^{2+} in hepatocytes [21] as well as in the Mz-ChA-1 cholangiocarcinoma cell line [22], and these agents caused a rapid increase in bile duct cells as well (n > 100 observations) [13]. Secretin (100 nM), which increases cAMP in cholangiocytes [3], did not increase Ca_i^{2+} in these cells (n = 5). These findings provide functional evidence for both muscarinic and P_{2U} nucleotide receptors on bile duct cells.

Mechanisms of Ca^{2+} signaling in bile duct cells

Ca_i^{2+} signals induced by stimulation of either muscarinic or P_{2U} receptors occur through $InsP_3$-mediated release of endogenous Ca^{2+} stores in most cell types [23]. A series of experiments was performed to investigate whether this mechanism similarly is responsible in the cholangiocytes [13]. First, bile duct cells were stimulated with ATP (10 μM) or ACh (10 μM), either in the presence or absence of 1.9 mM Ca^{2+} in the perifusing medium [13]. Increases in Ca_i^{2+} were elicited by either agonist, even in Ca^{2+}-free medium (Table 1). Thus, ATP- or ACh-induced Ca_i^{2+} signals result from mobilization of endogenous Ca^{2+} stores. Such stores generally depend upon a Ca^{2+}-ATPase to remain filled, and this Ca^{2+} pump is inhibited by thapsigargin [24]. We found that thapsigargin increased Ca_i^{2+} in the bile duct cells and prevented any further increase by subsequent

Table 1. Ca_i^{2+} signaling in bile duct cells in regular and Ca^{2+}-free medium.

	Single Ca_i^{2+} response, %	Ca_i^{2+} oscillations, %	n
ATP (10 μM):			
1.9 mM extracellular Ca^{2+}	20	80	25
Ca^{2+}-free medium	37	63	30
ACh (10 μM):			
1.9 mM extracellular Ca^{2+}	84	16	44
Ca^{2+}-free medium	84	16	38

Single responses include both sustained and transient increases in Ca_i^{2+}, whereas oscillations denote any type of repetitive response. Adapted from [13].

stimulation with ACh, while reducing subsequent, ATP-induced Ca_i^{2+} increases [13]. A series of microinjection studies were performed to determine whether these thapsigargin-sensitive Ca^{2+} stores are gated by the $InsP_3$ receptor. Heparin is a high-affinity specific antagonist of the $InsP_3$ receptor [25], while its de-*N*-sulfated form is not [26]. Therefore we injected individual bile duct cells within bile duct units with either heparin or de-*N*-sulfated heparin. None of 8 cells injected with heparin responded to subsequent stimulation with ATP (10 μM), whereas 60 percent (6/10) of cells injected with de-*N*-sulfated heparin responded. For comparison, 60 ± 21 percent of non-injected cells responded to ATP (Figure 1). Together, these findings suggest that ACh and ATP increase Ca_i^{2+} in bile duct cells via $InsP_3$-mediated release of endogenous Ca^{2+} stores.

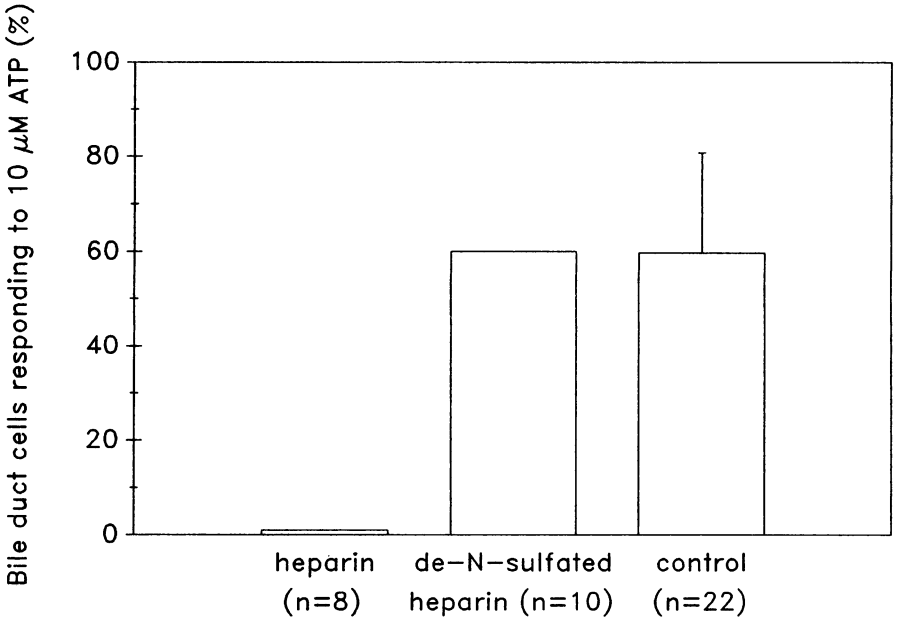

Figure 1. $InsP_3$ mediates ATP-induced Ca_i^{2+} signals in bile duct cells. The $InsP_3$ receptor antagonist heparin blocks ATP-induced Ca_i^{2+} signals in individual bile duct cells within isolated bile duct units, whereas de-*N*-sulfated heparin does not. For heparin and de-*N*-sulfated heparin, n refers to the number of cells injected, whereas for controls, n refers to the number of ductules observed.

Paracrine Ca^{2+} signaling from hepatocytes to bile duct cells

Bile ducts receive parasympathetic innervation by the vagus nerve [27], which is the likely source of ACh *in vivo*. However, the source of extracellular ATP that would stimulate hepatocytes or cholangiocytes is less clear. ATP is present in nanomolar to micromolar amounts in bile [28], suggesting that hepatocytes could be the source of this signaling molecule in liver. We examined this question directly in cultures of isolated hepatocytes and in co-cultures of isolated hepatocytes and bile duct cells [16]. Upon mechanical stimulation of individual isolated hepatocytes, an increase in Ca_i^{2+} was seen in the hepatocyte that was stimulated as well as in hepatocytes up to 300 μm away, including those not in physical contact with other hepatocytes [16]. The Ca_i^{2+} increase in the mechanically stimulated hepatocyte was not associated with uptake of propidium iodide, demonstrating that the Ca_i^{2+} signal was not the result of damage to the cell. The Ca_i^{2+} increase in neighboring hepatocytes could be retarded or blocked by the ATP/ADPase apyrase (50 U/ml), the P_2 receptor antagonist suramin (100 μM), or pretreatment with ATP to desensitize P_2 receptors [16]. Together, these findings suggest that this intercellular signaling among hepatocytes results from ATP secretion by the mechanically stimulated cell, followed by activation of P_2 receptors on its neighbors. Similarly, mechanical stimulation of isolated hepatocytes was followed by increases in Ca_i^{2+} in 15 out of 23 (i.e., 64 percent) co-cultured bile duct units, and these Ca_i^{2+} increases were blocked by apyrase as well [16]. Thus, hepatocytes have the ability to secrete nucleotides and, in sufficient quantity, to stimulate P_2 receptors on nearby hepatocytes and bile duct cells.

Ca^{2+} does not directly stimulate secretion in hepatocytes or bile duct cells

Since the above observations show that ATP and ACh increase Ca_i^{2+} in bile duct cells, and since patch clamp studies by others had identified Ca^{2+}-activated Cl^- channels in biliary cell lines [10, 11], we examined whether increases in Ca_i^{2+} stimulate secretion in isolated bile duct units [13]. For comparison, secretion in isolated hepatocyte couplets was examined as well. Ductules were examined for a 15 min control period, then for an additional 30 min while stimulated with ATP (10 μM; n = 7), ACh (10 μM; n = 10), or thapsigargin (2 μM; n = 8). Ductular secretion also was measured over 45 min in a separate, unstimulated control group. ACh increased luminal volume only marginally (p = .09) in the ductules, while ATP and thapsigargin had no significant effect on luminal volume, relative to the non-stimulated controls (Figure 2). In contrast, secretin, dibutyryl cAMP and forskolin each significantly increase luminal volume under these conditions (Figure 2) [15]. Secretin's effect on secretion is less pronounced than that of forskolin or dibutyryl cAMP, probably because secretin increases intracellular cAMP to a lesser extent. In separate studies, the effects of Ca_i^{2+} and cAMP on primary canalicular secretion were compared in isolated rat hepatocyte couplets. As in bile duct units, the cell-permeant cAMP analogs dibutyryl cAMP (100 μM) and 8-chlorophenyl-cAMP (100 μM) and the adenyl cyclase activator forskolin (50 μM) each stimulated secretion in the couplets (Table 2).

Table 2. Cyclic AMP but not Ca_i^{2+} stimulates secretion in isolated rat hepatocyte couplets.

Group	Bile flow (fl/min)	Volume change (%/min)	n
Dibutyryl cAMP*	10.3 ± 4.1	7.6 ± 5.1	13
8-chlorophenyl-cAMP*	13.0 ± 5.5	6.5 ± 2.8	11
Forskolin*	11.1 ± 5.3	6.9 ± 4.4	26
1,9-dideoxyforskolin	1.7 ± 2.2	2.3 ± 1.5	14
Vasopressin	<0.0	-28.7 ± 14.9	12
Control	3.8 ± 3.8	1.9 ± 1.6	27

*Increases in bile flow and percent change in volume are significant relative to controls ($p < .005$). Values are mean ± SD.

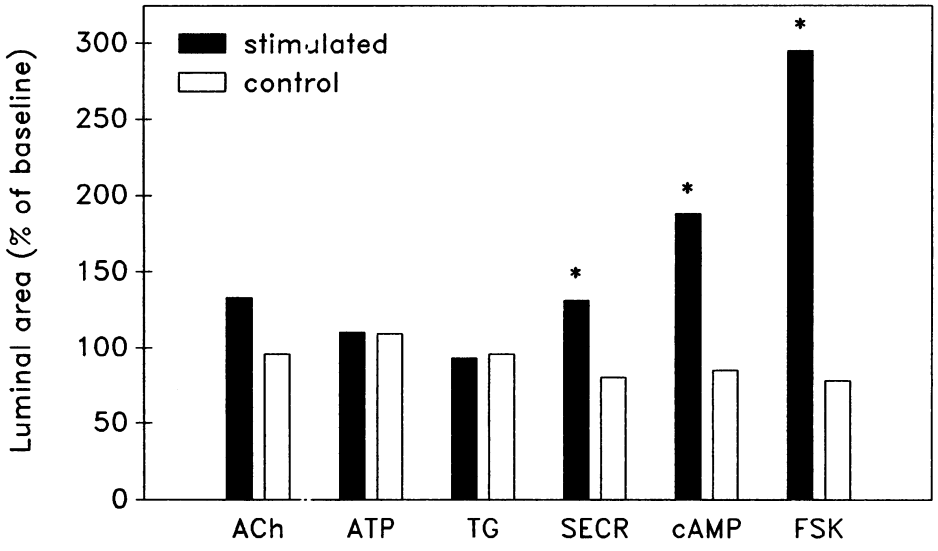

Figure 2. Increases in Ca_i^{2+} do not stimulate secretion in bile duct cells, relative to pair-matched unstimulated controls isolated from the same liver preparations. Values shown reflect the size of isolated bile duct lumens (measured after 30 min of stimulation), relative to their baseline values. Values for ACh (10 μM), ATP (10 μM), and thapsigargin (TG; 2 μM) are from Reference [13], while values for secretin (200 nM), dibutyryl cAMP (100 μM) + IBMX (50 μM), and forskolin (FSK; 10 μM) are from [15]. *Denotes $p < .01$ relative to controls.

This likely is due not only to activation of canalicular transporters, but to targeting and insertion of additional transporters to the canalicular membrane as well [29]. The cAMP-induced increases in volume over time were more modest in the couplets than in the ductules, perhaps because couplets were observed over a shorter time interval, and since the capacity of the couplet lumen to expand is likely far more limited than that of the ductule lumen. In contrast to bile duct units, hepatocyte couplets stimulated with a Ca_i^{2+} agonist (such as vasopressin; Table 2) displayed a rapid decrease in luminal volume over time. Previous studies also have reported that vasopressin and other Ca_i^{2+} agonists exert this effect in hepatocytes [12, 17], which results from Ca_i^{2+}-induced contraction of pericanalicular actin [21,30]. Thus, cAMP directly stimulates secretion in both hepatocytes and bile duct cells, whereas Ca_i^{2+} does not.

SUMMARY AND FUTURE DIRECTIONS

Ca_i^{2+} is a critical second messenger in virtually all types of cells. Here we reviewed which agonists increase Ca_i^{2+} in bile duct cells, as well as the mechanism by which these agonists act. We also provided evidence that increases in Ca_i^{2+} do not directly stimulate bile ductular secretion. What is the physiological effect of Ca_i^{2+} in these cells? Preliminary work by other groups suggests several possibilities: First, ACh-induced increases in Ca_i^{2+} potentiate secretin-induced increases in cAMP [31], so that Ca_i^{2+} may be a modifier of cAMP-mediated actions in bile duct cells. Second, the work reviewed here examined the effects of basolateral stimulation (with ATP or ACh) on ductular secretion. However, stimulation of the apical surface of the NRC bile duct cell line with ATP activates Cl^- channels in these cells [32], so that increases in Ca_i^{2+} may have different effects depending upon whether they result from apical or basolateral receptor stimulation.

Such differential actions of receptors have been described in other epithelia [33], but it remains to be demonstrated more directly whether apical exposure of bile ducts to ATP stimulates secretion. Finally, it has alternatively been hypothesized that the Ca^{2+}-activated Cl^- channels of biliary cells are important for volume regulation rather than secretion [34]. Although we have found that neither ACh nor ATP acutely alters cholangiocyte volume [13], it is unknown whether these Ca^{2+}-activated Cl^- channels are involved in regulatory changes in cell volume. It is likely that future work will more clearly define the physiologic role of Ca_i^{2+} and of Ca^{2+}-activated Cl^- channels in bile duct cells.

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