

IS VESICULAR EXOCYTOSIS AND MEMBRANE RECYCLING A MECHANISM FOR SECRETIN-INDUCED CHOLERESIS IN BILE DUCT EPITHELIUM?

Kato A, Gores GJ, LaRusso NF. Secretin Stimulates Exocytosis in Isolated Bile Duct Epithelial Cells by a Cyclic AMP-mediated Mechanism. *J Biol Chem* 1992; 267:15523-15529.

ABSTRACT

Intrahepatic bile duct epithelial cells, or cholangiocytes, contribute to bile secretion in response to hormones, including secretin. However, the mechanism by which secretin stimulates ductular bile flow is unknown. Since recent data in nonhepatic epithelia have suggested a role for exocytosis in fluid secretion, we tested the hypothesis that secretin stimulates exocytosis by isolated cholangiocytes. Cholangiocytes were isolated from normal rat liver by a newly described method employing enzymatic digestion and mechanical disruption followed by immunomagnetic separation using specific monoclonal antibodies, and exocytosis was measured using a fluorescence unquenching assay employing acridine orange. Secretin caused a dose-dependent (10^{-12} – 10^{-7} M) increase in acridine orange fluorescence by acridine orange-loaded cholangiocytes with a peak response at 10 min; the half-maximal concentration of secretin was 7×10^{-9} M. The secretin effect was inhibited by preincubation of cholangiocytes with colchicine (30% inhibition, $p < 0.05$) or trypsin (90% inhibition, $p < 0.001$); no inhibition was seen with lumicolchicine and heat-inactivated trypsin. Cholecystokinin, insulin, and somatostatin had no effect on fluorescence of acridine orange-loaded cholangiocytes; secretin had no effect on fluorescence of acridine orange-loaded hepatocytes or hepatic endothelial cells. Exposure of isolated cholangiocytes to secretin at doses that stimulated exocytosis caused a dose-dependent increase in cyclic AMP levels (218% maximal increase, $p < 0.05$); moreover, an analogue of cyclic AMP stimulated exocytosis by cholangiocytes. Secretin had no effect on intracellular calcium concentration using Fura-2-loaded cholangiocytes assessed by digitized video microscopy. Our results demonstrate, for the first time, that secretin stimulates exocytosis by rat cholangiocytes. The effect is cell- and hormone-specific, dependent on intact microtubules, on a protein(s) on the external surface of cholangiocytes, and on changes in cellular levels of cyclic AMP. The results are consistent

with the hypothesis that secretin-induced changes in bile flow may involve an exocytic process.

COMMENTS

Since secretin was discovered by Bayliss and Starling in 1902, it has been found to stimulate pancreatic and bile duct secretion in several mammalian species (1). The hormone interacts with specific receptors to activate adenylate cyclase and increase the cyclic AMP (cAMP) second-messenger system, leading to the secretion of water and bicarbonate (2, 3). Recent studies using radioactively labeled secretin demonstrated specific, trypsin-sensitive, high-affinity secretin binding sites on rat intrahepatic biliary epithelium. This finding correlates with the increases in ductular tissue mass in bile duct-ligated livers (2). However, the mechanism by which cAMP stimulates HCO_3^- secretion is not yet clear. In pig pancreatic duct cells and cholangiocytes, secretin decreases the number of intracellular vesicles, presumably through a colchicine-inhibitable exocytotic process that correlates with the secretin-induced increase in bile and bicarbonate secretion (4, 5).

In contrast to hepatocytes, studies of the mechanisms of biliary secretion in bile duct epithelium have been limited because they represent only 3% to 5% of liver cells and are difficult to isolate in sufficient purity and numbers (6, 7). However, bile duct epithelium (BDE) cell hyperplasia can be induced by bile duct ligation, and some progress has been made with this model (6, 7). Recently, however, BDE cells have been isolated from normal rat and human liver by several techniques, including immunomagnetic separation as in study by Kato, Gores and LaRusso under discussion here, permitting more detailed examination (8, 9).

In the article under discussion, the authors showed quite convincingly by applying an acridine orange (AO) unquenching assay method that secretin causes a dose-dependent, cell-specific, hormone-specific release of AO from BDE cells through a temperature- and microtubule-dependent exocytotic process. AO, a permanent weak base, has been used as a fluorescent probe for cytoplasmic acidic compartments such as vesicles or vacuoles because it is protonated and thus trapped and quenched in these acidic environments. Incorporation of AO into these vesicles or compartments can be monitored by loss of cellular fluorescence. In contrast, release of AO from the acidic compartment results in an increase in fluorescence by unquenching; this is the effect observed after secretin in the study under comment.

What, then, does this hormone-induced fluorescence mean? The authors demonstrate that it is not due to cell

lysis; the viability of these cells did not change. Nor is the increase in AO fluorescence due to a collapse of the pH gradient of acidic vesicles; secretin does not alter the intracellular pH of BDE cells, as we recently showed using BCECF-AM (10). Moreover, the pH inside the vesicles does not change, as assessed by a pH-sensitive fluorescence ratio method using FITC-dextran in the Kato study. Therefore it is reasonable to conclude that the dose-dependent, secretin-induced increase in AO fluorescence was due to its release from the vesicles. Furthermore, the process is temperature and microtubule dependent, providing further evidence for an exocytotic process. As also shown, secretin induces exocytosis in these normal isolated BDE cells in a dose-dependent (up to 5×10^{-8} mol/L), trypsin-sensitive, cell-specific, and secretin analog-blocking manner, presumably through cAMP-mediated pathways and without changes in cytosolic free calcium.

Although these studies demonstrate that secretin can stimulate exocytosis of acidic vesicles in these isolated rat BDE cells, it remains unclear whether these events are also responsible for secretin stimulation of bicarbonate choleresis. In addition, in the isolated BDE cells from the rat, cell polarity is lost; thus the specific domain where this exocytotic process occurs is unknown.

In the BDE cells from the intact pig, exocytosis occurs predominantly at the basolateral membrane rather than at the apical membrane (4). A high arterial PCO_2 increases the number of cytoplasmic vesicles in this bile duct epithelium and is paralleled by changes in the degree of secretin-induced ductular choleresis in this species (4, 5). Moreover, the clearance of cytoplasmic vesicles induced by secretin is associated with significant but reversible plasma membrane surface area changes resulting in contraction of apical membrane and considerable expansion of the basolateral domain (4, 5). These findings suggest recycling of intracellular membrane between apical and basolateral membranes (4).

Kato, Gores and LaRusso also propose a "membrane microdomain recycling hypothesis" to explain secretin-induced choleresis. This concept of membrane recycling is not new and began with the pioneering work of Brown and Goldstein on receptor-mediated endocytosis of low-density lipoprotein receptor (11). The hypothesis states that specific plasma membrane components such as transport proteins or channels are inserted and removed, respectively, by exocytosis and endocytosis of specialized transport vesicles (4, 11, 12). Virtually all eukaryotic cells regulate the composition of their plasma membrane through this process (11-13). Kato, Gores and LaRusso propose that ductular bicarbonate secretion is mediated by shuttling of particular membrane transport proteins or channels between the plasma membrane of BDE cells and the "storage vesicles," although the roles of the acidic intracellular vesicles in the BDE cells are unknown.

In the turtle bladder, when proton secretion is stimulated, specialized cytoplasmic vesicles carrying H^+ -ATPase migrate to and fuse with the apical plasma membrane, resulting in further increases in proton secretion by these newly inserted proton pumps (12). In the intercalated cells of renal collecting tubule,

$\text{HCO}_3^-/\text{Cl}^-$ exchangers are retrieved from the apical domain in response to reductions in the pH of their environment (13). In contrast, in rat hepatocyte couplets an exposure to HCO_3^- -containing medium increases both the activity and apical targeting of $\text{HCO}_3^-/\text{Cl}^-$ exchangers (14).

Can such membrane recycling adequately account for secretin-induced bicarbonate choleresis in BDE cells? Although more research will be needed before this question can be answered, several additional experimental findings may be relevant. First, studies in the pig suggest that $\text{N,N}'$ -dicyclohexylcarbodiimide, a potent H^+ -ATPase inhibitor, reduces bicarbonate secretion without any effect on hepatocyte bile production (15). Furthermore, preliminary studies in amiloride-pretreated, microdissected intrahepatic bile ducts from pig showed that bafilomycin A_1 , a specific blocker of vacuolar type H^+ -ATPase, blocked the secretin-dependent pH_i recovery from an acute acid load (16). This secretin-dependent pH_i recovery was not blocked by Na^+ substitution with choline, suggesting that secretin stimulates a sodium-independent vacuolar H^+ -ATPase in pig BDE cells (16). These findings, if confirmed in the rat, would be consistent with basolateral extrusion of acid-containing vesicles in bile duct epithelium because a basolateral acid extrusion process should be countered by apical acid loading mechanisms (e.g., bicarbonate excretion). Recent work from our laboratory indicates that secretin stimulates $\text{Cl}^-/\text{HCO}_3^-$ exchange in isolated normal BDE cells and that this occurs without a change in intracellular pH (10). Neither Na^+/H^+ exchange nor $\text{Na}^+/\text{HCO}_3^-$ symport is stimulated by secretin, leaving open the possibility that a H^+ -ATPase fulfills the role of an acid extruder as shown in pig.

In addition, somatostatin may also play a role in the regulation on the exocytosis in BDE cells. Preliminary data suggest that somatostatin inhibits secretin-induced choleresis in bile duct-ligated rats but not in sham-operated rats (17). Somatostatin also inhibits secretin-induced exocytosis in isolated BDE cells, suggesting a counterregulatory role in the control of bile ductular secretion (17).

Despite these intriguing observations, vesicle recycling cannot be the only regulatory mechanism. Secretin also stimulates opening of chloride channels in isolated rat BDE cells; this appears to be necessary for the activation of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Preliminary electrophysiological studies using patch-clamp techniques provide additional evidence that secretin can open low-conductance chloride channels in excised inside-out patches by means of cAMP-mediated stimuli (18). Thus it is likely that secretin stimulates bicarbonate choleresis by more than one mechanism. This interesting study should point the way to further research in this area.

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ACUTE HEPATITIS A: AN ENDANGERED SPECIES

Werzberger A, Mensch B, Kuter B, Brown L, Lewis J, Sitrin R, Miller W, et al. A controlled trial of formalin-inactivated hepatitis A vaccine in healthy children. *N Engl J Med* 1992;327:453-457.

ABSTRACT

Background: Although inactivated hepatitis A vaccine is known to be well tolerated and immunogenic in healthy children and adults, its efficacy has yet to be established.

Methods: To evaluate the efficacy of the hepatitis A vaccine in protecting against clinically apparent disease, we conducted a double-blind, placebo-

controlled trial in a Hasidic Jewish community in upstate New York that has had recurrent outbreaks of hepatitis A. At the beginning of a summer outbreak, 1037 healthy seronegative children 2 to 16 years of age were randomly assigned to receive one intramuscular injection of a highly purified, formalin-inactivated hepatitis A vaccine or placebo. A case was defined by the presence of typical signs and symptoms, a diagnostic increase in IgM antibody to hepatitis A, and a serum concentration of alanine aminotransferase at least twice the upper limit of normal. Cases occurring ≥ 50 days after the injection were included in the evaluation of efficacy. The children were followed for a mean of 103 days.

Results: A total of 519 children received vaccine, and 518 received placebo. The vaccine was well tolerated, with no serious adverse reactions. From day 50 after the injection, 25 cases of clinically apparent hepatitis A occurred in the placebo group and none in the vaccine group ($P < 0.001$), confirming that the vaccine had 100 percent protective efficacy. Before day 21, seven cases occurred in the vaccine group and three cases in the placebo group. After that time, there were no cases among vaccine recipients and 34 cases among placebo recipients.

Conclusions: The inactivated purified hepatitis A vaccine that we tested is well tolerated, and a single dose is highly protective against clinically apparent hepatitis A.

COMMENTS

Although the history of viral hepatitis spans approximately 4,000 yr, it was not until the last five decades that important scientific advances identified the causative agents. Over the centuries, hepatitis A virus (HAV) has been suspected in or associated with large hepatitis outbreaks; the most recent one was reported in Shanghai, where more than 300,000 jaundice cases were observed (1).

Since the isolation of HAV in 1973 (2), several investigators have pursued the preparation of a vaccine to prevent HAV infection. Scientific communications about live-attenuated and inactivated prototype HAV vaccines started appearing in 1978 and are expected to continue to be published. Indeed, many of these products have shown promising results with regard to safety and immunogenicity in human subjects (3-7). However, we know of only two highly successful HAV vaccine efficacy trials. The work by Werzberger et al. is the first to be published in the scientific literature. The second trial tested a comparable inactivated hepatitis A vaccine, prepared with the HM175 strain of HAV; the efficacy rate was 97% (8). In essence, Werzberger et al. describe the success of an inactivated hepatitis A vaccine when administered as a single dose to a population at risk.

The human trial was conducted in a population of children aged 2 to 16 yr. These children were residents of the Kiryas Noel Hasidic Jewish community in Monroe, NY and were known to have a 3% annual incidence of acute hepatitis A. After provision of informed consent by their parents, children were enrolled in the study if they had undetectable serum antibodies to HAV (anti-HAV). The study was a randomized,