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Role of the Neuropeptide, Bombesin, in Bile Secretion

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Since ancient times, bile secretion has been considered vital for maintaining health. One of the main functions of bile secretion is gastric acid neutralization with biliary bicarbonate during a meal or Pavlovian response. Although the liver has many extrinsic and intrinsic nerve innervations, the functional role of these nerves in biliary physiology is poorly understood. To understand the role of neural regulation in bile secretion, our recent studies on the effect of bombesin, a neuropeptide, on bile secretion and its underlying mechanisms will be reviewed.

Using isolated perfused rat livers (IPRL) from both normal and 2 week bile duct ligated rats, as well as hepatocyte couplets and isolated bile duct units (IBDU) from normal rat livers, bombesin was shown to stimulate biliary bicarbonate and fluid secretion from bile ducts. Detailed pH studies indicated that bombesin stimulated the activity of Cl^-/HCO_3^- exchanger, which was counterbalanced by a secondary activation of electrogenic Na^+/HCO_3^- symport. Quantitative videomicroscopic studies showed that bombesin-stimulated fluid secretion in IBDU was dependent on Cl^- and HCO_3^- in the media, anion exchanger(s), Cl^- and K^+ channels, and carbonic anhydrase, but not on the microtubular system. Furthermore, this bombesin response is inhibited by somatostatin but not substance P. Finally, studies of secondary messengers in isolated cholangiocytes and IBDU indicated that bombesin had no effect on intracellular cAMP, cGMP, or Ca^{++} levels in cholangiocytes.

These results provide evidence that neuropeptides such as bombesin can directly stimulate fluid and bicarbonate secretion from cholangiocytes by activating luminal Cl^-/HCO_3^- exchange, but by different mechanisms from those established for secretin. These findings, in turn, suggest that neuropeptides may play an important regulatory role in biliary transport and secretion. Thus, this neuropeptidergic regulation of bile secretion may provide a plausible mechanism for the bicarbonate-rich choleresis seen with meals or Pavlovian response.

INTRODUCTION

As noted by ancient Babylonians and Greeks who considered bile as one of cardinal humors, vital for health, bile secretion serves a fundamental role in our well being. It aids gastric acid neutralization, toxin or waste material excretion, and fat digestion. Although the Pavlovian response in gastric acid secretion is well understood, not much is known about such responses in bile secretion. Traditionally, secretin, identified by Bayliss and Starling in 1902, was thought to be the main regulator of biliary bicarbonate secretion. However, *in vivo* studies showed that bicarbonate-rich bile secretion increased not only

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[b] *Abbreviations:* IPRL, isolated perfused rat liver; IBDU, isolated bile duct unit; GRP, gastrin-releasing peptide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; VIP, vasoactive intestinal peptide.

with feeding [1] but also with sham feeding [2], suggesting the importance of neural regulation in postprandial choleresis.

Since the late nineteenth century, it has been recognized that the liver is richly supplied with nerves [3]. Although considerable interspecies variations exist, intrinsic innervation by fibers predominantly follows the vascular and biliary structures. Some nerve fibers may reach the hepatic lobule where they form a network around hepatocytes and extend into the sinusoids [4, 5]. Hepatic nerves have both efferent and sensory functions in carbohydrate and lipid metabolism, regulation of the hepatic microcirculation, and osmo- and chemoreception. However, their function in bile secretion is poorly understood, partly due to inadequate study models and to interspecies variations in bile secretory response to nerve stimulation.

In addition, abundant neuropeptidergic nerves are also present in portal triads and along the sinusoids [4, 5]. Immunoreactivity to various peptides within ganglion cells and nerves in the hepatic plexus and in the intrahepatic periarterial spaces suggests that these peptides are intrinsic to these structures [4] and thus might regulate the function of the bile duct epithelium or the hepatic vasculature [5]. Nevertheless, the functional significance of these hepatic peptidergic nerves in biliary physiology and bile secretion is not clear.

To further study the functional role of these neuropeptidergic nerves in bile secretion, direct effects of particular neuropeptides on bile secretion can be studied. However, there are many potential complicating problems in studying and interpreting the role of neuropeptides in bile secretion due to the intrinsic complexities of bile secretion that involve both hepatocytes with bile acid dependent and independent mechanisms, and subsequent modifications at the level of cholangiocytes. It is also difficult to determine whether these effects are directly from these neuropeptides or indirectly from neurally mediated release of hormones, alterations in perfusion, and/or metabolic changes. Furthermore, many of these neuropeptides may also interact with other previously known secretagogues such as glucagon, cholecystokinin, and secretin.

To understand the role of neuropeptides in the regulation of biliary secretion, recent findings on one of neuropeptides, bombesin, will be reviewed and summarized in this paper. Bombesin is a tetradecapeptide initially isolated from the skin of the European frogs *Bombina bombina* and *Bombina variegata variegata* [6, 7]. Subsequently, its mammalian homologue, gastrin-releasing peptide (GRP)[b], was isolated from mammalian gut extracts [8]. Bombesin and GRP, exclusively found in nervous tissue [9-11], have identical biological actions and similar potencies [12, 13]. These peptides increase gastric secretion [14, 15] and gut motility [16, 17], stimulate gallbladder contraction [17, 18], and stimulate the release of numerous peptides such as neurotensin, motilin, insulin, cholecystokinin, secretin, and glucagon [19-22].

Although bombesin/GRP induces a bicarbonate-rich biliary secretion in dog and pig [22-25], the underlying mechanism or site and mode of action is poorly understood. To approach these questions, both *ex vivo* and *in vitro* systems were used to eliminate influences from the release of hormones and from nerve interactions. Individual components of bile secretion were studied one at a time to determine the site of action. Various pH and quantitative videomicroscopic studies were employed to further characterize the underlying ion transport mechanisms involved in the bombesin-stimulated biliary secretion. In addition, cAMP, cGMP, and calcium levels in isolated cholangiocytes were also assessed to examine the signal transduction pathways of bombesin in cholangiocytes.

REVIEW OF RESULTS

Isolated perfused rat liver

To study the direct effect of bombesin on bile secretion, without interference from other potential secretagogues released by bombesin, the isolated perfused rat liver (IPRL) model was used. An infusion of bombesin (100 nM) in IPRL with or without taurocholate supplementation changed neither total bile flow nor bile acid output, indicating that bombesin had no significant effects on either bile-acid-dependent or independent bile secretion [26, 27]. However, bombesin (100 nM) infusion in bile-acid-depleted IPRL significantly increased bile pH (+0.06 unit), bile HCO_3^- concentration (+3.9 mM), and HCO_3^- output (+3.8 nmole/min/g of liver), indicating a stimulatory effect on bicarbonate secretion [26, 27].

To investigate the origin of the increased HCO_3^- secretion, a 2 week-bile duct ligated rat model was used to enhance the contribution of the bile duct epithelium to total bile flow. This model has been used previously to study the bile ductular biliary secretion in rats [28, 29]. As expected, bombesin (100 nM) infusion increased bile flow (+0.05 ml/min/g of liver) in bile duct ligated rats with a greater increase in bile pH (+0.10 unit), bile HCO_3^- concentration (+5.8 mM), and HCO_3^- output (+6.0 nmole/min/g of liver) [26, 27]. These findings in the IPRL model indicate that bombesin stimulated biliary bicarbonate secretion in normal rats but that the effect was more marked in bile duct ligated rats with bile duct proliferation, suggesting an importance of bile ductular component.

Hepatocyte couplet

To directly examine whether bombesin stimulated hepatocyte cannalicular bile secretion, quantitative videomicroscopic measurements of the hepatocyte couplet lumen were used to monitor secretory response as previously used with other secretagogues [30, 31]. While infusion of the positive control, glucagon (100 nM), increased the luminal area by 60 percent, an administration of bombesin (10-100 nM) to hepatocyte couplets had no effect, supporting the results from IPRL studies where bombesin had no effect on net secretion from hepatocytes [26, 27].

Isolated bile duct unit

Because the bile ductular component accounts for less than 10-15 percent of total bile flow, changes in bile ductular secretion may not always be reflected in changes in total bile flow as discussed above. To directly study the effect of bombesin on bile ductular secretion and to reconfirm the results obtained with the IPRL model, the novel functional, polarized IBDU model was utilized [32].

Administration of bombesin (10 nM) to IBDUs increased their luminal areas by 30-40 percent in 30 min, as assessed by quantitative videomicroscopy, indicating net fluid secretion from bile ducts [27, 33]. Comparing this secretory response with other neuroendocrine peptides showed that bombesin (~40 percent) is less potent than secretin (~60 percent) or VIP (~90-100 percent) when administered at the same dose (100 nM) [26, 27, 33, 34]. A dose-response study from 0 -1000 nM showed that IBDUs responded to as little a dose as 0.1 nM (10^{-10}M) and saturated at ~10-100 nM [27, 33]. Furthermore, when a specific, competitive bombesin receptor inhibitor, (Tyr^4-D-Phe^{12})-bombesin, was coinfused, an almost complete but reversible inhibition of the bombesin secretory response was observed [27], suggesting an involvement of specific bombesin receptors.

Microinjection of the IBDU lumen with BCECF-Dextran, a cell impermeable pH-sensitive fluorescent dye, showed that bombesin significantly increased the luminal pH (+0.11 pH unit) [27]. This finding indicated that the increase in luminal area of the IBDU also accompanied by an increase in luminal pH, suggesting increased bicarbonate secretion at the level of bile ducts.

Detailed pH studies employing BCECF 440/495 dual ratio methods were used to examine the underlying ion transport mechanisms involved in this bombesin-stimulated biliary secretion. Three major ion transporters have been characterized previously in cholangiocytes and are important for pH regulation and for biliary secretion: two acid extruders (Na^+/H^+ exchanger and Na^+/HCO_3^- symporter) and one acid loader (Cl^-/HCO_3^- exchanger) [29, 35]. Bombesin (10 nM) did not affect basal pH_i nor the activities of Na^+/H^+ exchange or Na^+/HCO_3^- symport, as assessed by the recovery rates after a standard NH_4Cl acid load in HEPES or in Krebs-Ringer bicarbonate in the presence of amiloride [33, 36, Manuscript submitted]. However, bombesin (10 nM) significantly increased Cl^-/HCO_3^- exchange activity, reflected by maximal rates of alkalinization of pH_i after Cl^- removal and of pH_i recovery after Cl^- readmission [33, Manuscript submitted]. Depolarization of cholangiocytes increased basal pH_i and the activity of Cl^-/HCO_3^- exchanger, suggesting that an electrogenic Na^+/HCO_3^- symport might function as a counter-regulatory pH_i mechanism [33, 36, Manuscript submitted]. These results suggest that the bombesin-stimulated bicarbonate-rich biliary secretion is mediated by the increased Cl^-/HCO_3^- exchange activity, which is counter-balanced by the secondary activation of electrogenic Na^+/HCO_3^- symport to maintain pH_i [Manuscript submitted].

In order to further characterize the underlying mechanisms of the bombesin response, the effect of omitting bicarbonate or chloride from the perfusion medium was studied. Omitting bicarbonate by substituting Krebs-Ringer bicarbonate with HEPES medium, or replacing chloride with gluconate resulted in almost complete inhibition of the secretory response to bombesin, suggesting dependence of the bombesin response on bicarbonate and chloride [26, 27]. Bombesin-stimulated secretion was also completely inhibited by co-administration of H_2-DIDS (0.5 mM) and significantly inhibited by the chloride channel blocker, NPPB (10 μM), the potassium channel blockers, $BaCl_2$ (1 mM) and triethylammonium ion (10 μM), and by the carbonic anhydrase inhibitor, acetazolamide (100 μM) [36, Manuscript submitted]. However, unlike studies with secretin or VIP, colchicine (10 μM) pretreatment had no significant effect on the bombesin response, when compared to β-lumicolchicine (10 μM) [36, Manuscript submitted]. These findings indicate that bombesin-stimulated secretion is dependent on Cl^- and HCO_3^- in the media, anion exchanger(s), Cl^- and K^+ channels, and carbonic anhydrase, but not on the microtubular system.

To understand the modulatory effects of other neuroendocrine peptides on the bombesin response, quantitative videomicroscopy was used. Coadministration of somatostatin (100 nM) inhibited the bombesin (10 nM) response while the albumin carrier did not affect luminal expansion [27, 33]. However, withdrawal of somatostatin promptly reversed this inhibition, suggesting no sustained inhibitory effect. In contrast, coadministration of substance P (10-100 nM), which has been shown to have an anticholeretic effect in the in-vivo dog model, did not have any significant effect on bombesin response [27, 33].

Isolated cholangiocytes

To characterize the signal transduction pathway involved in the bombesin response in bile duct epithelium, pure (>90-95 percent γ–glutamyl transferase positive) cholangiocytes, prepared by immunomagnetic isolation methods [37, 38], were used. The cAMP and cGMP levels in isolated cholangiocytes measured after stimulation with bombesin (0.1-1000 nM) did not change, while the appropriate positive controls, secretin and forskolin for cAMP, and sodium nitroprusside for cGMP, increased significantly above baseline [36]. Preliminary measurements of calcium using Fura-2 dual ratio methods also suggested that bombesin (10 nM) did not change intracellular Ca^{++} levels in cholangiocytes.

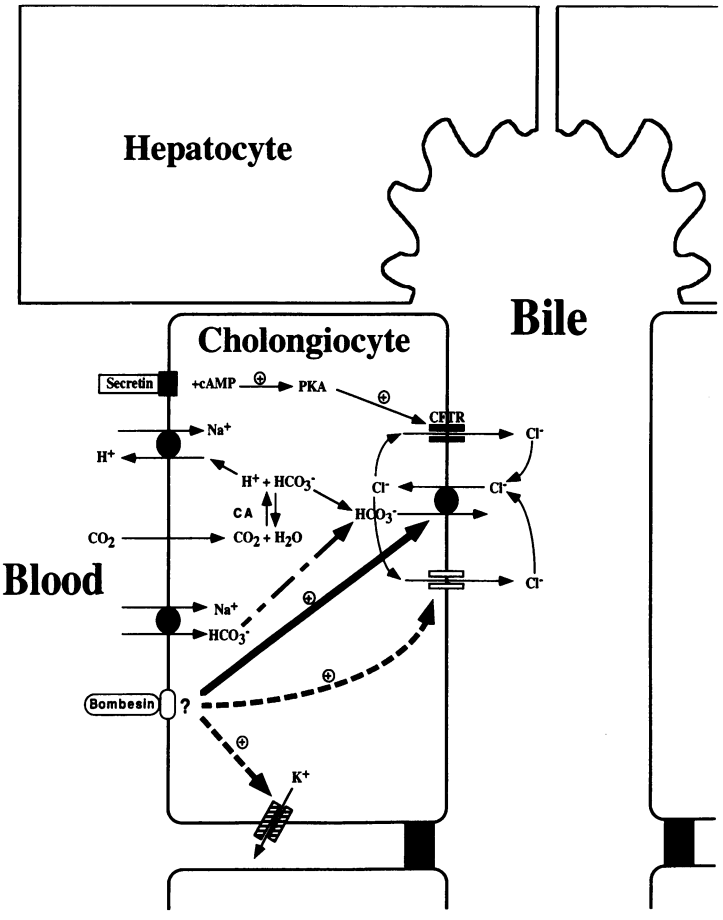

Figure 1. Bombesin-stimulated secretion in bile duct epithelium.

DISCUSSION

These studies demonstrate that the neuropeptide bombesin, through specific bombesin receptors, can directly stimulate biliary bicarbonate and fluid secretion in the rat, mainly by acting at the level of cholangiocytes. As shown in Figure 1, this bombesin-stimulated biliary bicarbonate and fluid secretion is mediated by activation of the Cl^-/HCO_3^- exchange activity in cholangiocytes, which is presumably counterbalanced by secondary increase in the activity of the electrogenic Na^+/HCO_3^- symporter to keep pH_i constant. Furthermore, the dependence of these processes on the functions of chloride and potassium channels suggests that the opening of these channels may provide the driving force for the Cl^-/HCO_3^- exchanger and the electrogenic Na^+/HCO_3^- symporter by regulating Cl^- concentration gradient and membrane potential (Figure 1).

Unlike secretin, bombesin response is not dependent on microtubules nor is it associated with increased cAMP levels in isolated cholangiocytes. These findings indicate that bombesin has a distinctly different underlying mechanisms from secretin to induce HCO_3^- secretion from cholangiocytes. Currently, the signal transduction pathway mediating the bombesin-stimulated secretion in cholangiocytes is not known.

Study of modulating roles of other neuroendocrine peptides on the bombesin response revealed that somatostatin, but not substance P, had direct inhibitory effects on bombesin-stimulated biliary secretion from bile ducts. However, in the in-vivo setting, these inhibitory neuropeptides may also have additional indirect modulatory effects by affecting the release or metabolism of bombesin from nerve terminals.

These studies suggest that central and/or hepatic nerves may regulate secretion by releasing neuropeptides which can activate specific receptors on cholangiocytes thereby modulating biliary transport and secretion. Bombesin/GRP is almost exclusively confined to nerve fibers [9-11] and GRP-like immunoreactivity was identified within ganglion cells and nerves in the hepatic plexus and in the intrahepatic periarterial spaces, suggesting an intrinsic origin for this peptide [4]. Moreover, nerve cell bodies with bombesin-like immunoreactivity are also localized in hypothalamic and medullary nuclei in the central nervous system that are involved in the regulation of autonomic functions [39]. Therefore, autonomic and/or intrinsic hepatic neural pathways might regulate biliary secretion through neuropeptides such as bombesin by producing a bicarbonate-rich choleresis to counteract the increase in acid loads during food digestion or Pavlovian response.

SUMMARY

Bombesin-stimulated fluid and HCO_3^- secretion in cholangiocytes is mediated by activation of Cl^-/HCO_3^- exchanger, which is presumably balanced by electrogenic Na^+/HCO_3^- symporter to maintain pH_i. This process is dependent on Cl^- and K^+ channels, anion exchanger, and carbonic anhydrase, but not on microtubules. The signal transduction system involved in this bombesin response is unknown.

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