Stimulatory effects of bilirubin on amylase release from isolated rat pancreatic acini

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Hirohata, Yoshihide, Masatoshi Fuji, Yoshinori Okabayashi, Yoshikuni Nagashio, Mitsu Tashiro, Issei Imoto, Toshiharu Akiyama, and Makoto Otsuki. Stimulatory effects of bilirubin on amylase release from isolated rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 282: G249–G256, 2002; 10.1152/ajpgi.00429.2002.—Considered to be an etiologic factor of acute pancreatitis, hypersecretion of pancreatic juice and digestive enzymes is often associated with hyperbilirubinemia. We explored the intracellular mechanisms through which bilirubin affects pancreatic exocrine secretory function by examining the effect of bilirubin on isolated rat pancreatic acini. Bilirubin stimulated amylase release in a concentration- and time-dependent manner, significantly increasing amylase release at concentrations >5 mg/100 ml and after 15 min of incubation. Coincubation of bilirubin with vasoactive intestinal polypeptide, 8-bromo-cAMP, or A-23187 had a synergistic effect on amylase release, whereas coincubation with CCK-8, carbamylcholine, or 12-0-tetradecanoylphorbol 13-acetate had an additive effect. Bilirubin did not affect acinar cAMP content or Ca2+ efflux. Intracellular Ca2+ pool depletion had no influence on bilirubin-evoked amylase release. The protein kinase C (PKC) inhibitors staurosporine and calphostin C partially but significantly inhibited bilirubin-stimulated amylase release, whereas the PKA inhibitor H-89 did not. The tyrosine kinase (TK) inhibitor genistein, phospholipase A2 (PLA2) inhibitor indoxam, and PLC inhibitor U-73122 also inhibited amylase release. Bilirubin significantly translocated PKC activity from the cytosol to the membrane fraction and activated TK in cytosol and membrane fractions. These results indicate that bilirubin stimulates amylase release by activating PKC and TK in rat pancreatic acini and that PLC and PLA2 partly mediate this process.

signal transduction; protein kinase C; tyrosine kinase; phospholipase C; phospholipase A2

ACUTE PANCREATITIS IS OFTEN associated with hyperbilirubinemia observed in patients of obstructive jaundice (11, 16, 17, 33), fulminant hepatic failure (10, 15, 18, 32), and acute massive hemolysis (9, 14). These clinical observations may suggest that hyperbilirubinemia, whether in conjugated or unconjugated form, affects exocrine pancreatic function. In fact, hypersecretion of pancreatic juice and/or digestive enzymes has been observed in patients with liver cirrhosis (11, 33) and obstructive jaundice (17). Moreover, hyperbilirubinemia is considered to be one of the etiologic factors of biliary pancreatitis (16). Hyperbilirubinemia-associated pancreatic hypersecretion and injury of the exocrine pancreas in acute pancreatitis may be accounted for in several possible ways. Liver damage may decrease the degradation or excretion of the circulating gastrointestinal peptides such as CCK and secretin (7, 19, 38), resulting in the persistent stimulation of the exocrine pancreas. An alternative interpretation is based on the negative feedback control of CCK release by intraluminal bile acids or other bile components, shown to be operative in humans as well as in experimental animals (12, 13, 22–24). Because bile secretion into the intestine is almost blocked in obstructive jaundice, it may counteract a postulated negative feedback control of CCK secretion by bile acids and thereby increase circulating plasma CCK levels (12, 13, 22–24). Indeed, endogenous CCK release is tonically inhibited by bile in the human intestine (22). In addition, bile acids increase the sensitivity of the exocrine pancreas to secretagogues of diacylglycerol (DAG) formation and subsequent activation of protein kinase C (PKC) (36). However, little is known about the direct effect of bilirubin that accumulates in the circulation and/or periacinar space of the exocrine pancreas in jaundice.

In the present study, we examined the effect of bilirubin on basal and secretagogue-stimulated pancreatic exocrine secretion and the intracellular mechanisms through which bilirubin affects pancreatic exocrine secretory function in isolated rat pancreatic acini in vitro.

MATERIALS AND METHODS

Chemicals. Bilirubin, carbamylcholine chloride, A-23187, 12-0-tetradecanoylphorbol 13-acetate (TPA), 8-bromo-cAMP (8-brcAMP), forskolin, staurosporine, IBMX, HEPES, benza-

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midine, phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, Nonidet P-40, U-73122, EDTA, EGTA, and soybean trypsin inhibitor (SBTI) (type 1-S) were purchased from Sigma (St. Louis, MO). Calphostin C was obtained from Kyowa Medics (Tokyo, Japan), and N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinolinesulfonamide (H-89) was from Seikagaku Kogyo (Osaka, Japan). Genistein was purchased from Calbiochem-Novabiochem (San Diego, CA), and chromatographically purified collagenase (type CLSPA) was from Cooper Biochemical (Malvern, PA). Eagle's minimum medium albumin supplement was obtained from Gibco Life Technologies (Grand Island, NY), and 45CaCl2 (1.5 GBq/mg Ca) and PKC assay kit were from Amersham International (Buckinghamshire, UK). Bovine plasma albumin (fraction V) was purchased from Armour Pharmaceutical (Phoenix, AZ), and CCK-8 and vasoactive intestinal polypeptide (VIP) were from Peptide Institute (Osaka, Japan). cAMP (Phoenix, AZ), and CCK-8 and vasoactive intestinal polypeptide (VIP) were from Peptide Institute (Osaka, Japan).

Preparation of isolated pancreatic acini. Male Wistar rats (Kyudo, Kumamoto, Japan), weighing 250–280 g, were used in the experiments. The animals were kept at 23 °C in a 12:12-h light-dark cycle with free access to water and a standard laboratory diet (CE-2, Kyudo).

Isolated rat pancreatic acini were prepared according to a method described previously (29). The acini were suspended in HEPES-buffered Ringer (HR) solution containing (in mM) 10 HEPES, 128 NaCl, 1 NaH2PO4, 4.7 KCl, 1.28 CaCl2, 11.1 glucose with 0.1 mg/ml SBTI, 5 mg/ml bovine plasma albumin, and Eagle's minimum medium albumin supplement.

Amylase release. After a 30-min recovery incubation, the following experiments were performed. The acini were suspended in fresh HR at a density of 0.35–0.45 mg acinar protein/ml. Aliquots (2 ml) were distributed into 25-ml polycarbonate flasks. Amylase release during a 30-min incubation with various concentrations of bilirubin or various secretagogues was determined using a procedure described previously (29). The effects of bilirubin on amylase release evoked by various concentrations of receptor-mediated or bypassing agents were determined similarly. Moreover, the effects of the PKC inhibitors staurosporine (37) and calphostin C (21), the PKA inhibitor H-89 (5), the tyrosine kinase (TK) inhibitor genistein (25), the phospholipase C (PLC) inhibitor U-73122 (43), and the PLA2 inhibitor indoxam (42) on bilirubin-evoked amylase release were determined. Acini were preincubated with 1 μM staurosporine, 0.1 μM calphostin C, 30 μM H-89, 1 μM U-73122, 1 μM indoxam, or 300 μM genistein for 30 min at 37 °C. After centrifugation, acini were resuspended in fresh incubation medium containing the same concentration of inhibitors, and then further incubated with or without 5 mg/100 ml bilirubin for 30 min at 37 °C. The time-course of amylase release was examined by incubating the isolated acini either with or without 5 mg/100 ml bilirubin, and the amylase activity in the medium was determined at various times. Amylase activity was determined by a chromogenic method using the Phadebas amylase test (4).

To deplete the intracellular Ca2+ pool, the acini were incubated with 1 nM CCK-8 in Ca2+-free HR containing 1 mM EGTA for 30 min at 37 °C. At the end of this pretreatment, the acini were washed twice with fresh Ca2+-free HR containing 1 mM EGTA and further incubated with 5 mg/100 ml bilirubin, 100 μM CCK-8, or 3 μM carbamylicholine in Ca2+-free HR (plus 1 mM EGTA) for 30 min at 37 °C.

Viability of acini. The viability of the acini after a 30-min incubation with bilirubin was evaluated by determining lactate dehydrogenase (LDH) release into the incubation medium and by a trypan blue dye exclusion test. LDH activity was determined using the method of Wroblewski and LaDue (41).

Intracellular cAMP content. Acini were incubated with 5 mg/100 ml bilirubin and/or 0.1 μM VIP in the presence of 1 mM IBMX for 30 min at 37 °C. At the end of the incubation period, 1 ml of sample was centrifuged at 3,000 g for 10 s. One milliliter of ice-cold 0.1 N HCl was added to each acinar pellet, the pellet was sonicated for 10 s, and then cAMP and protein concentrations were determined. cAMP content in the acini was measured by RIA, according to the method of Ogami et al. (27). Protein concentration was determined using the method of Bradford (2). Cellular cAMP was calculated relative to the protein concentration of the acinar pellet.

Ca2+ efflux. Efflux of 45Ca2+ from the isolated pancreatic acini was measured as reported previously (28). The acini were preincubated for 30 min at 37°C in HR, and 45CaCl2 (74 kBq/ml) was then added and incubation continued for another 60 min. At the end of this loading period, the acini were centrifuged, washed once with ice-cold HR, resuspended in prewarmed HR, and further incubated in the presence or absence of 5 mg/100 ml bilirubin at 37°C. Samples were taken at 0, 5, and 30 min and centrifuged at 10,000 g for 20 s. Radioactivity in the medium was determined by liquid scintillation counting. For each time period, the 45Ca2+ remaining in the acini was calculated as the percentage of the 45Ca2+ present at the beginning of the washout period.

*P < 0.01, significantly different vs. no bilirubin; **P < 0.01, significantly different vs. control at the corresponding time.

Fig. 1. A: concentration dependence of amylase and lactate dehydrogenase (LDH) release from isolated rat pancreatic acini. In the presence of various concentrations of bilirubin, acini were incubated for 30 min at 37°C. B: time dependence of amylase release stimulated by bilirubin (5 mg/100 ml) from isolated rat pancreatic acini. Amylase and LDH release into the medium was expressed as %total enzyme activity initially present in the acini. Values are means ± SE of 4–5 separate experiments. *P < 0.01, significantly different vs. no bilirubin; **P < 0.01, significantly different vs. control at the corresponding time.
PKC and TK enzyme activities. Acini were incubated with the appropriate agents for 30 min at 37°C. At the end of the incubation period, 1 ml of sample was centrifuged at 3,000 g for 20 s. The acinar pellet was washed once with 1 ml of ice-cold solution A (pH 7.5, 50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% (wt/vol) β-mercaptoethanol, 10 mM benzamidine, and 50 μg/ml PMSF). The pellet was then resuspended in 1 ml of ice-cold solution A, followed by sonication for 10 s with a probe-type sonicator. The resulting suspension was centrifuged at 100,000 g for 60 min at 4°C. The supernatant was saved for assay of PKC or TK enzyme activity (cytosolic fraction), while the pellet was further homogenized with 1 ml of ice-cold solution A containing 1% Nonidet P-40 and left on ice for 1 h. The homogenate was centrifuged at 100,000 g for 60 min at 4°C, and the supernatant was taken for assay of PKC or TK enzyme activity (membrane fraction). PKC enzyme activity was measured using an enzyme assay kit, whereas TK enzyme activity was measured as reported previously using poly(L-Glu,L-Tyr) (4:1) as a substrate (34). The reaction mixture in a final volume of 50 μl contained 2.5 μM Tris-HCl, 2.5 μM MgCl₂, 0.5 mM orthovanadate, 0.02% Triton X-100, 3 mM ATP, 0.4 μCi [γ-32P]ATP, and 100 μg Glu-Tyr polymer. The reaction was initiated by adding each sample. The reaction was terminated by applying 20 μl of the reaction mixture onto 3-cm² Whatman no. 3 filter paper.

Statistics. Results are expressed as means ± SE. The statistical significance of differences between means was assessed by Student’s t-test for unpaired samples. Differences of P < 0.05 were considered significant.

RESULTS

When isolated rat pancreatic acini were incubated with bilirubin for 30 min, amylase secretion increased in a concentration-dependent manner (Fig. 1A). A significant increase was observed at concentrations >5 mg/100 ml. Under these conditions, bilirubin at all concentrations except 10 mg/100 ml failed to increase LDH release into the medium (Fig. 1A). This suggests that all the acini remained intact at bilirubin concentrations <10 mg/100 ml, which was confirmed by the viability of acini using the trypan blue dye exclusion test (data not shown). Thus bilirubin at a concentration of 2.5 or 5 mg/100 ml was used in the following experiments. As shown in Fig. 1B, 5 mg/100 ml bilirubin caused time-dependent amylase release, and a significant amylase release was seen after 15 min of incubation.

Because bilirubin is known to bind to albumin (3), we studied the effect of the albumin concentration in the incubation medium on bilirubin-stimulated amylase release. Albumin concentration was reduced from the standard concentration of 0.5% to 0.05%. With the reduced concentration of albumin, the concentration-response curve to bilirubin shifted leftward more than twofold, whereas 2.5 mg/100 ml bilirubin induced a significant amylase release (6.8 ± 0.1% vs. 1.8 ± 0.1% for basal release, P < 0.01, n = 4). On the other hand, the responsiveness to 100 pM CCK-8 and 1 nM VIP was essentially the same, regardless of the concentration of albumin in the incubation medium (100 pM CCK-8, 15.4 ± 0.6% in 0.05% albumin vs. 15.7 ± 0.4% in 0.5% albumin; 1 nM VIP, 3.5 ± 0.6% in 0.05% albumin vs. 3.7 ± 0.4% in 0.5% albumin, n = 5).

We then studied the interaction between bilirubin and secretagogues acting through the receptors on acini. In isolated rat pancreatic acini, CCK-8 (100 pM) 15.7 ± 0.6% in 0.5% albumin; 1 nM VIP, 3.5 ± 0.6% in 0.05% albumin vs. 3.7 ± 0.4% in 0.5% albumin, n = 5).

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Table 1. Effect of bilirubin on amylase release stimulated by various secretagogues in isolated rat pancreatic acini

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>Control</th>
<th>Bilirubin</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCK-8 (100 pM)</td>
<td>15.7 ± 0.6%</td>
<td>17.2 ± 0.6%</td>
</tr>
<tr>
<td>Carbamylcholine (1 μM)</td>
<td>16.5 ± 0.6%</td>
<td>16.5 ± 0.6%</td>
</tr>
<tr>
<td>Secretin (1 nM)</td>
<td>3.5 ± 0.3%</td>
<td>10.1 ± 0.9%†</td>
</tr>
<tr>
<td>VIP (1 nM)</td>
<td>4.1 ± 0.4%</td>
<td>12.4 ± 1.0%†</td>
</tr>
<tr>
<td>A-23187 (10 nM)</td>
<td>4.0 ± 0.2%</td>
<td>9.8 ± 0.8%†</td>
</tr>
<tr>
<td>TPA (1 μM)</td>
<td>6.2 ± 0.4%</td>
<td>9.4 ± 0.4%*</td>
</tr>
<tr>
<td>8-BrcAMP (100 μM)</td>
<td>4.1 ± 0.4%</td>
<td>9.8 ± 1.2%†</td>
</tr>
<tr>
<td>8-BrcAMP, 8-bromo-cAMP</td>
<td>8-BrcAMP, 8-bromo-cAMP</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–6 separate experiments. Pancreatic acini were incubated with various agents in the presence or absence of 5 mg/100 ml bilirubin for 30 min at 37°C. Amylase release is expressed as %total amylase activity initially present in the acini. VIP, vasointestinal polypeptide; TPA, 12-O-tetradecanoylphorbol 13-acetate; 8-BrcAMP, 8-bromo-cAMP. *Significantly different vs. respective control; †Significantly different vs. additive response.
pancreatic acinar cells in stimulating amylase release. For both CCK-8 and carbamylcholine, the addition of bilirubin produced an additive pattern of amylase release without a shift in the concentration-dependent curve (Fig. 2). Bilirubin did not alter the sensitivity of pancreatic acini to CCK-8 and carbamylcholine in terms of the concentrations of these secretagogues required to stimulate half-maximal amylase release. To confirm that bilirubin does not activate either the CCK or muscarinic cholinergic receptors, we studied the effects of the CCK receptor antagonist loxiglumide (10 μM) and the muscarinic cholinergic receptor antagonist atropine (10 μM). At concentrations completely blocking the effects of their respective agonists, neither loxiglumide nor atropine had an inhibitory effect on bilirubin-stimulated amylase release (data not shown). Therefore, bilirubin-stimulated amylase release does not mediate either CCK or muscarinic cholinergic receptors. We also examined the effect of bilirubin on amylase release stimulated by receptor-bypassing agents. In the presence of bilirubin, phorbol ester TPA caused an additive increase in amylase release, whereas the Ca²⁺ ionophore A-23187 had a synergistic effect on amylase release (Table 1). The insignificant increase of LDH release and exclusion of trypan blue indicated that synergistic release caused by bilirubin plus A-23187 was not due to acinar cell damage (data not shown). These results suggest that bilirubin may stimulate amylase release via the DAG-PKC system but not by a Ca²⁺-mediated pathway.

To examine the effect of bilirubin on amylase release evoked by the cAMP pathway, we incubated acini with increasing concentrations of either secretin, VIP, forskolin, or 8-BrcAMP in the presence or absence of 5 mg/100 ml bilirubin. In contrast to CCK-8 or carbamylcholine, the addition of bilirubin to secretin or VIP caused a synergistic potentiation of amylase release. Bilirubin also potentiated the action of cAMP-mediated secretagogues such as forskolin and 8-BrcAMP that have postreceptor mechanisms (Table 1 and Fig. 3). We also measured the intracellular cAMP concentration to examine whether bilirubin acts as a adenylate cyclase stimulator or a phosphodiesterase inhibitor. Isolated acini were incubated with 1 mM IBMX in the presence or absence of 5 mg/100 ml bilirubin for 30 min. Bilirubin had no influence on the basal cAMP concentration or the VIP-stimulated increase in cellular cAMP [1.45 ± 0.03 vs. 1.56 ± 0.09 pmol/mg protein for control vs. 5 mg/100 ml bilirubin alone, respectively, not significant (NS); 16.72 ± 1.13 vs. 17.21 ± 1.36 pmol/mg protein for 10 nM VIP vs. 10 nM VIP + 5 mg/100ml bilirubin, respectively, NS, n = 5].

To further confirm that bilirubin does not stimulate amylase release via the Ca²⁺ pathway, we measured Ca²⁺ efflux from acinar cells. Isolated pancreatic acini preloaded with labeled Ca²⁺ were incubated in the presence or absence of 5 mg/100 ml bilirubin. Bilirubin did not alter the Ca²⁺ efflux (91 ± 1.1% vs. 89.9 ± 1.1% for control vs. 5 mg/100 ml bilirubin remaining at 5 min, NS; 68.7 ± 2.8% vs. 69.3 ± 4.4% for control vs. 5
EFFECT OF BILIRUBIN ON EXOCRINE PANCREAS

Table 2. Effects of protein kinase inhibitors on bilirubin-stimulated amylase release from isolated rat pancreatic acini

<table>
<thead>
<tr>
<th>Amylase Release, %initial content</th>
<th>Control</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8 ± 0.1</td>
<td>5.4 ± 0.2*</td>
</tr>
<tr>
<td>Staurosporine (1 μM)</td>
<td>1.7 ± 0.1</td>
<td>3 ± 0.2†</td>
</tr>
<tr>
<td>Calphostin C (0.1 μM)</td>
<td>1.7 ± 0.1</td>
<td>2.2 ± 0.1*†</td>
</tr>
<tr>
<td>Genistein (300 μM)</td>
<td>1.8 ± 0.1</td>
<td>3.4 ± 0.2*†</td>
</tr>
<tr>
<td>Calphostin C and genistein (300 μM)</td>
<td>1.9 ± 0.1</td>
<td>2 ± 0.4†</td>
</tr>
<tr>
<td>H-89 (30 μM)</td>
<td>1.8 ± 0.1</td>
<td>5.5 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4–6 separate experiments. Pancreatic acini were preincubated for 30 min at 37°C without or with staurosporine, calphostin C, H-89, or genistein. Acini were then incubated for 30 min at 37°C without or with each inhibitor in the presence of 5 mg/100 ml bilirubin. Amylase release is expressed as %total amylase activity initially present in the acini. *Significantly different vs. control (none). †Significantly different vs. bilirubin alone.

mg/100 ml bilirubin remaining at 30 min, NS, n = 5). We also depleted the intracellular Ca2+ pool by preincubating acini with CCK-8 in the presence of EGTA. After this treatment, the effects of CCK-8 or carbachol were completely abolished, whereas the effect of bilirubin was not reduced (Fig. 4). Therefore, the action of bilirubin is independent of intracellular Ca2+.

Secretagogues for exocrine pancreatic secretion stimulate amylase release by activating various protein kinases including PKA, PKC, and TK (39). We therefore examined the role of these kinases in bilirubin-stimulated amylase release. Amylase release was measured after incubating acini with bilirubin in the presence of the PKA inhibitor H-89 (5), the PKC inhibitors staurosporine (37) and calphostin C (21), and the TK inhibitor genistein (25). H-89 has been shown (5) to be a potent inhibitor of PKA activity, with complete inhibition of activity attained at a concentration of 30 μM. As shown in Table 2, however, H-89 at this concentration had no influence on bilirubin-stimulated amylase release. On the other hand, both 1 μM staurosporine and 0.1 μM calphostin C partially but significantly inhibited bilirubin-induced amylase release. The TK inhibitor genistein at a concentration of 300 μM also partially but significantly inhibited bilirubin-evoked amylase release. Moreover, bilirubin-stimulated amylase release was completely inhibited by simultaneous treatment with calphostin C and genistein. Therefore, activation of both PKC and TK mediates amylase release by bilirubin stimulation. We then investigated the effects of bilirubin on the subcellular distribution of PKC and TK. Bilirubin at a concentration of 2.5 mg/100 ml caused a significant redistribution of PKC enzyme activity from the cytosol to membrane fraction (Fig. 5) and activated TK enzyme activity in both the cytosol and membrane fraction (Fig. 6).

Phosphatidylinositol-specific PLC and PLA2 are thought to be the important membrane effectors involved in the pancreatic signal transduction system (39). Furthermore, PLC activates both PKC and TK (34), and PLA2 activates PKC (1). When treating bilirubin-stimulated acini with PLC inhibitor U-73122 or PLA2 inhibitor indomethacin, both 1 μM U-73122 and 1 μM indomethacin partially but significantly inhibited bilirubin-evoked amylase release (Fig. 7).

DISCUSSION

It is well known that jaundice is often associated with hypersecretion of pancreatic juice and enzymes (17) and with acute pancreatitis (9, 10, 14, 15, 18, 32). Several mechanisms have been suggested to possibly induce pancreatic hypersecretion in jaundice: an increase in circulating gastrointestinal peptide such as CCK and secretin due to impaired metabolism in the diseased liver (7, 19, 38); an increase in the circulating...
CCK levels resulting from a decrease in bile in the duodenum (12, 13, 22–24); or an increase in the sensitivity of pancreatic acinar cells to secretagogues such as CCK and secretin (36). However, the mechanism of direct action of bilirubin on the exocrine pancreas remains unclear.

In the present study, we showed that bilirubin can enhance amylase release by acting either alone or in concert with secretagogues that accumulate intracellular cAMPs, including secretin, VIP, forskolin, and 8-BrcAMP, a membrane-permeable analog of cAMP. Bilirubin potentiated amylase release in response to all these stimuli and failed to increase intracellular cAMP contents. However, the PKA inhibitor H-89 did not inhibit bilirubin-stimulated amylase release. Therefore, bilirubin seems to act on a pathway distal to the activation of PKA or on a different pathway that interacts with a cAMP-dependent signal transduction mechanism.

Because cytosolic Ca2+ mobilization in pancreatic acinar cells causes the potentiation of stimulated amylase release when combined with secretagogues that activate the cAMP pathway (34), we examined whether bilirubin acts on the Ca2+ mobilization mechanism. Because our data showed that bilirubin had no effect on 45Ca2+ efflux from pancreatic acini, Ca2+ mobilization does not appear to account for the major action of bilirubin. However, bilirubin may have some effect on Ca2+ mobilization, because the action of bilirubin was additive with CCK, carbachol, and TPA. Therefore, the present study indicates that the amylase-releasing effect of bilirubin is independent of intracellular Ca2+.

Activation of PKC by DAG is the other important intracellular messenger system involved in enzyme secretion from pancreatic acinar cells (30). TPA may exert its effect on enzyme secretion through PKC activation while not affecting Ca2+ or cyclic nucleotide metabolism (8). TPA also exerts sustained amylase release in pancreatic acini (20, 31). Ca2+ and DAG also have been shown (8, 35) to work synergistically on amylase release from pancreatic acini. Taken together with the present data showing that the action of bili-
rubin on amylase release is sustained and synergistic with the Ca\(^{2+}\) ionophore, it is conceivable that bilirubin stimulates amylase release by activating PKC. Indeed, the PKC inhibitors staurosporine and calphostin C significantly inhibited bilirubin-stimulated amylase release, although they did not completely abolish it. Therefore, it seems that other effectors or intracellular messengers in stimulus-secretion coupling are involved in amylase release evoked by bilirubin.

Staurosporine inhibits not only PKC but also TK activity (39), and it inhibited bilirubin-evoked amylase release more effectively than calphostin C. This suggests a potential role of tyrosine phosphorylation in the regulation of pancreatic acinar cell secretion (25). The TK inhibitor genistein also caused a partial but significant inhibition of bilirubin-stimulated amylase release. Thus bilirubin also stimulates amylase release by activating TK. However, it is unclear whether bilirubin also stimulates other effectors or messengers involved in amylase release besides PKC and TK, because neither PKC nor TK inhibitors completely abolished the action of bilirubin.

We therefore measured PKC and TK enzyme activities in cytosolic and membrane fractions from pancreatic acini. Because bilirubin caused a significant redistribution of PKC enzyme activity from the cytosol to membrane fraction and a significant activation of TK in both fractions, it is suggested that activation of PKC and TK participates in the stimulation of amylase release brought about by bilirubin. Although we did not confirm which PKC isoform is activated by bilirubin, it is conceivable that bilirubin stimulates amylase release via Ca\(^{2+}\)-independent PKC isoforms because the effect of bilirubin was independent of intracellular Ca\(^{2+}\).

Bilirubin is generally considered a lipophilic substance, and it binds to phospholipids on the pancreatic acinar cell membrane (6, 26). Williams and Yule (40) have suggested that not only polyphosphoinositides but also phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and their metabolites play an important role in the actions of gastrointestinal peptides in a number of cell types such as pancreatic acinar cells. In addition, phosphatidylcholine-specific PLC and PL\(_A_2\) are thought to be the important membrane effectors involved in the pancreatic signal transduction system (40). Therefore, it is conceivable that bilirubin influences membrane fluidity and thus changes the effect of secretagogues on the exocrine pancreas or affects a specific protein neighboring the receptor rather than the receptor itself, because of the ability of bilirubin to bind to the lipid membrane. We also confirmed the effects of the inhibitors of these phospholipases on bilirubin-evoked amylase release, finding that both PLC and PL\(_A_2\) inhibitors partially but significantly inhibit bilirubin-evoked amylase release.

According to the diazo method of analysis, >90% of the bilirubin used in this experiment was the unconjugated form. We were unable to determine whether conjugated bilirubin has the same effects as unconjugated bilirubin on pancreatic acinar cells due to the lack of an available preparation of conjugated bilirubin. It is possible, however, that unconjugated bilirubin exerts its effects on the exocrine pancreas, because jaundice due to hemolytic diseases as well as liver and biliary tract disease can induce acute pancreatitis (9, 10, 14, 15, 18, 32). Our study suggests a possible mechanism through which bilirubin induces pancreatic secretion and pancreatic injury by demonstrating the unique amylase-releasing effects of the bilirubin that accumulates in the circulation and/or periacinar space in patients with jaundice.

In the present study, we showed that (unconjugated) bilirubin stimulates amylase release and this action may be mediated by activating the membrane effectors, including PLC and PL\(_A_2\), and by subsequently activating the PKC and TK pathway.

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