Secretion of Intrinsic Factor from Cultured Rat Gastric Chief Cells

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Intrinsic factor (IF) is a vitamin B_{12} binding protein that is secreted from the gastric mucosa. We tested secretagogues which stimulate IF secretion in rat gastric perfusion and found that carbachol and cholecystokinin octapeptide (CCK-8) stimulated secretion, but histamine and tetragastrin did not. To confirm these results, we examined IF secretion from isolated rat chief cells. For this purpose, we established an enzyme immunoassay (EIA) using an avidin–biotin peroxidase complex to measure small amounts of IF. To prepare an anti-rat IF, IF was isolated from the stomach, and was injected into a rabbit for immunization. Rat gastric chief cells were isolated from the gastric mucosa with Dispase and a Percoll gradient centrifugation, and were cultured. We examined the effects of chemicals by adding them to culture dishes of chief cells in a CO_2 incubator. Released IF in culture medium was determined by EIA. Carbachol, CCK-8 and secretin stimulated IF secretion from cultured chief cells, while histamine and tetragastrin did not; Forskolin and A23187 also stimulated the secretion. We concluded that carbachol and CCK-8 stimulated IF secretion *via* an increase of intracellular Ca^{2+} concentration and that secretin did so *via* a cAMP accumulation.

Keywords intrinsic factor; secretion; gastric chief cell; carbachol; cholecystokinin; secretin

Intrinsic factor (IF) is a vitamin B_{12} binding glycoprotein, which is secreted from the gastric mucosa. Vitamin B_{12} which is a general name of cobalamin (Cbl) derivatives, is known as an anti-pernicious anemia factor and important as the coenzyme of the methylmalonyl CoA mutase and methionine synthase in mammals.[1] There are many recent reports dealing with the relationship between Cbl deficiency and nervous disorders[2] or AIDS.[3] It is known that Cbl ingested in food binds to R-binder which is a Cbl binding protein in saliva, and is then transferred to the duodenum. Soon after digestion of R-binder by pancreatic juice, IF immediately binds to Cbl and is transferred to the ileum. A complex of IF and Cbl binds to its receptor on the plasma membrane of the ileum, and Cbl is absorbed.[4] Although IF is necessary to absorb Cbl, little is known of this substance. Amino acid sequence of rat IF was determined from the cDNA sequence.[5] Generally, IF is secreted from gastric parietal cells of the human,[6] dog,[7] rabbit and guinea pig.[8] However, the content of IF in chief cells of the rat and mouse is reportedly much greater than that in parietal cells.[7] Though there is a report that IF was secreted from dispersed rat gastric mucosal cells,[9] the mechanism of IF secretion from chief cells is not yet known. We earlier reported that some secretagogues of pepsinogen secretion from chief cells are different from those of gastric acid secretion from parietal cells.[10] In this paper, we confirm what kind of cells contain IF, and examine what kinds of chemicals stimulate IF secretion from the gastric mucosa and chief cells.

MATERIALS AND METHODS

Materials Cyanocobalamin (CN-Cbl), hydroxocobalamin (OH-Cbl) acetate and histamine dihydrochloride were purchased from Wako Pure Chemical Industries (Osaka). ^{14}CN-Cbl was made from OH-Cbl substituted with K^{14}CN (ICN Biomedicals, Costa Mesa, U.S.A.). Sepharose 4B, Sephadex G-75 and Percoll were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Immobilon-PVDF transfer membrane was purchased from Japan Millipore Ltd. (Tokyo). Carbamylcholine chloride (carbachol), rat IF as the standard and forskolin were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Dispase I, and cholecystokinin octapeptide (CCK-8) and secretin were from Godo Shusei Co. (Tokyo) and Peptide Institute Inc. (Osaka). All other reagents were of the best commercial quality available.

Purification of IF from Rat Gastric Mucosa Supernatant of rat stomach homogenate was applied on an affinity chromatography made from Sepharose 4B conjugating CN-Cbl with 3-chloropropylamine as a spacer.[11] After washing the column, IF was eluted with 0.1 M phosphate buffer containing 7.5 M guanidine hydrochloride. Dialyzed and lyophilized IF fraction was purified on a Sephadex G-75 column.

Measurement of IF by Enzyme Immunoassay (EIA) Antiserum was prepared in the Japanese White rabbit by immunization of purified rat IF with Freund's complete adjuvant. An IgG fraction was separated by addition of $(NH_4)_2SO_4$ and DEAE-cellulose column chromatography. Then, we made up the EIA with an avidin–biotin peroxidase complex to measure rat IF: using this rabbit anti-rat IF antibody as a solid phase and biotinylated antibody, IF was measured with the avidine-conjugated peroxidase blocking nonspecific coloring by a treatment with normal rabbit serum.

Rat Gastric Perfusion Technique Under urethane anesthesia, rats were operated according to the method of Ghosh and Schild.[12] Phosphate buffered saline (pH 6.8) was used as a perfusion solution, since IF is possibly digested by pepsin in an acidic condition. Each chemical was injected through the femoral vein. Cbl binding protein was measured with a Cbl-binding protein capacity assay (CBPCA).[13] One unit of CBPCA corresponds to the amount of IF that binds to 1 ng of Cbl. Briefly, a mixture of ^{14}CN-Cbl and an aliquot of perfusate, collected every 20 min were incubated for 20 min, and then for

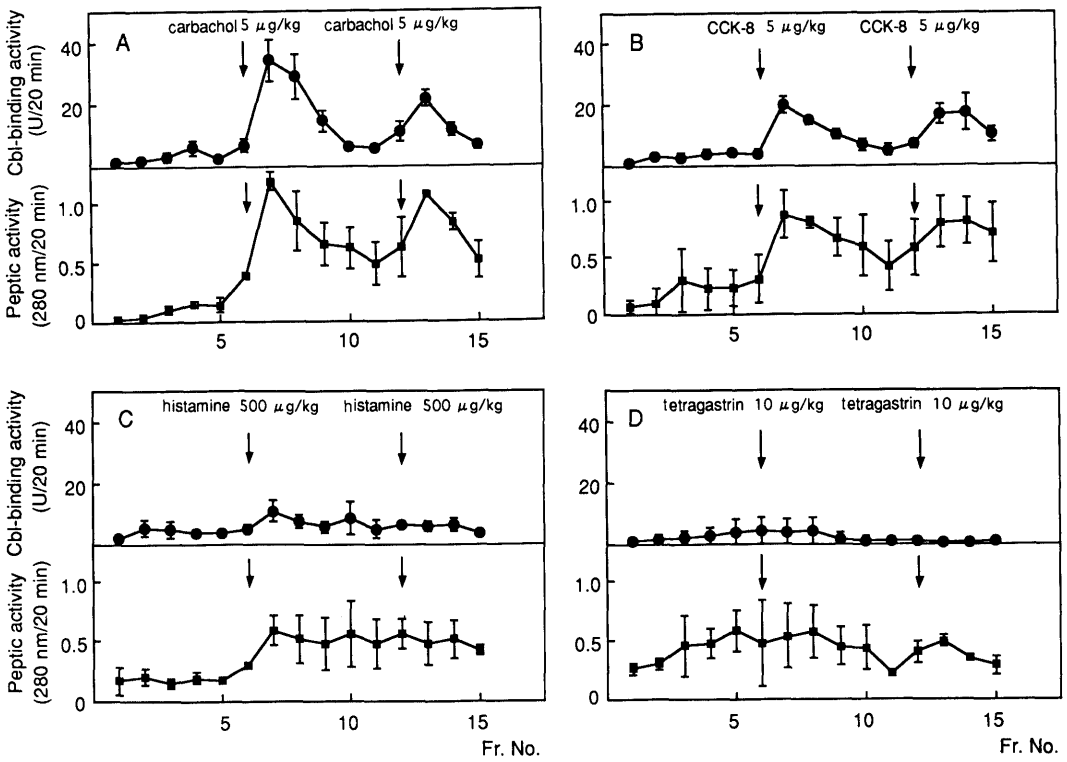

Fig. 1. Effects of Carbachol, CCK-8, Histamine and Tetragastrin on IF and Pepsinogen Secretion in Rat Gastric Perfusion

Isotonic phosphate buffer (pH 6.8) was perfused at the rate of 0.05 ml/min, and a perfusate was collected every 20 min. The indicated doses of chemicals (A, carbachol 5 μg/kg; B, CCK-8 5 μg/kg; C, histamine 500 μg/kg; D, tetragastrin 10 μg/kg) were injected intravenously at the arrows. Each value is mean ± S.E. from three experiments.

15 min with activated charcoal. After centrifugation, the radio-activity of the supernatant was measured with a scintillation counter. Peptic activity was measured by the modified method of Anson and Mirsky[14] and expressed as the total absorbance at 280 nm per min of the perfusate.

Isolation and Culture of Rat Gastric Chief Cells Gastric chief cells from the rat stomach were prepared by Percoll gradient centrifugation after dispersion from the reversed stomach with dispase I, as described previously.[15] Obtained chief cells were cultured in a mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 (1 : 1) containing 10% fetal calf serum on collagen coated plastic dishes at 37 °C in a CO_2 incubator.

RESULTS

Cbl-Binding Protein Secretion in Rat Gastric Perfusion Effects of some gastric acid secretagogues on secretion of Cbl- binding protein were tested in a rat gastric perfusion *in vivo*. Effective doses of carbachol, CCK-8, histamine and tetragastrin were used for stimulation. The same dose of the chemical was given twice at a 2 h interval, and the responses were compared. Secretion of Cbl-binding protein and pepsinogen was stimulated by carbachol and CCK-8 (Fig. 1A, B), but was little stimulated by histamine and tetragastrin (Fig. 1C, D). The responses of IF and pepsinogen secretion to the second dosage were smaller than those to the first. Especially, no IF secretion was seen to be induced by the second dosage of histamine or tetragastrin.

Isolation of IF from the Rat Stomach The purified IF showed one band in the gel of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at the same position of the standard rat-IF. One mole of the purified IF bound 1.25 mol of CN-Cbl stoichiometrically. This IF contains 8.9% of hexose determined by the method of Dubois *et al.*[16] Antiserum against the purified rat IF bound to the standard rat IF and rat stomach homogenate at the same position on a sheet of Immobilon to which the gel of SDS-PAGE was transferred (Fig. 2).

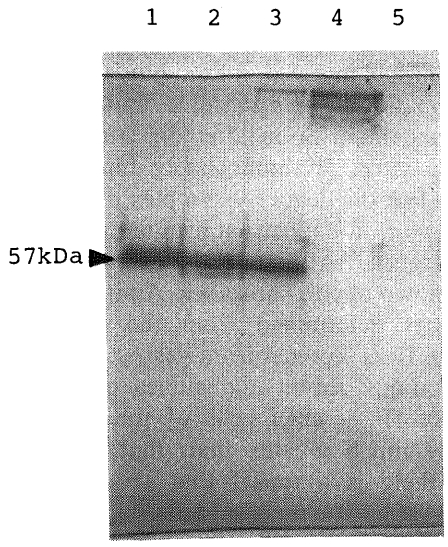

Fig. 2. Western Blot of IFs, Serum and Tissue Homogenates of the Stomach and Liver

The membrane on which a gel of SDS-PAGE was transferred was immersed in the diluted antiserum against rat IF, and the bound antibody was detected with biotinylated rabbit anti-IF antibody and the avidin-conjugated peroxidase. Line 1, standard rat IF; line 2, purified rat IF; line 3, supernatant of 10% rat stomach homogenate; line 4, rat serum; line 5, supernatant of 10% rat liver homogenate.

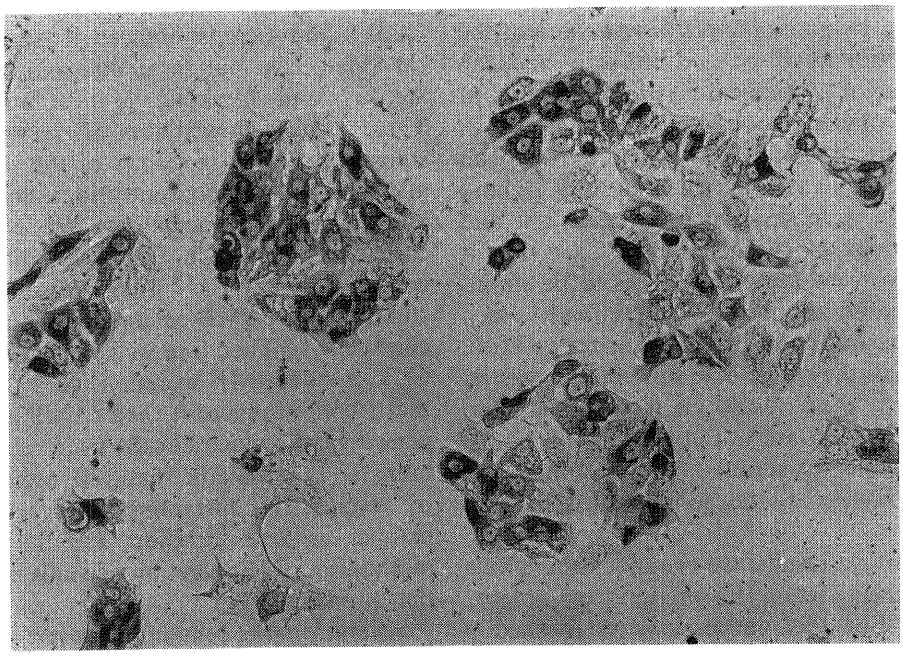

Fig. 3. Immunohistochemical Study of IF in Cultured Chief Cells

After fixation with 10% formalin, the cells were incubated with diluted rabbit anti-IF antiserum (1:100) at 4 °C for 18 h. They were then incubated with biotinylated goat anti-rabbit IgG antibody at 37 °C for 30 min, then with the avidin-conjugated peroxidase at 37 °C for 30 min. The color was developed with 3-amino-9-ethylcarbazole in 50 mM acetate buffer (pH 5.0). (×200).

TABLE I. Contents of IF and Cbl-Binding Protein in Some Tissues and Serum

Tissue	IF ($\times 10^{-4}$ g/g tissue)	Cbl-binding protein (U/g tissue)
Submaxillary gland	0.004±0.000	1.710±0.262
Stomach (corpus)	6.353±1.424	1.502±0.157
Liver	2.372±0.514	0.168±0.043
Spleen	0.078±0.013	0.156±0.054
Kidney	0.599±0.193	0.073±0.009
Serum	0.413±0.063	0.081±0.014

Each value is the mean±S.E. from three experiments.

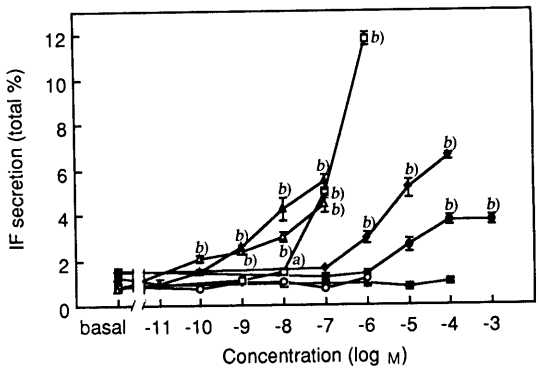

Fig. 4. Dose–Response Curves for the Effects of Some Secretagogues of IF Secretion from Rat Cultured Chief Cells

IF secretion is expressed as % of total IF that was estimated after freezing and thawing of cultured chief cells. Each value is mean±S.E. from four experiments. Symbols: ●, carbachol; ■, histamine; ▲, CCK-8; ○, tetragastrin; □, A23187; △, secretin; ◆, forskolin. *a*) $p<0.05$, *b*) $p<0.01$.

Also, some other bands were found at shorter migration distances on the lines of rat serum and stomach homogenate. No distinct band was observed, however, on the line of liver homogenate.

IF Contents in Chief Cells and Some Tissues More than 80% of the chief cell fraction content was chief cells by immunohistochemical staining with rabbit anti-rat pepsinogen antiserum (data not shown). Immunohistochemical study showed that all cultured cells seemed to be positive to IF, although the intensity of staining in cells was different (Fig. 3). IF content of the chief cell fraction measured by EIA was 165±10 ng/10^5 cells, and that of a residual fraction was 30±4 ng/10^5 cells. IF contents of some tissues were measured with EIA and CBPCA (Table I), and the values were estimated independently. A large amount of Cbl-binding protein was found in the corpus of stomach and the submaxillary gland.

IF Secretion from Cultured Chief Cells The effects of chemicals on IF secretion from cultured rat chief cells were examined (Fig. 4). After addition of the chemical to the culture medium for 1 h, IF in an aliquot of each incubation medium was measured with EIA. Carbachol, CCK-8 and secretin, at a concentration above 1×10^{-5}, 1×10^{-9} and 1×10^{-10} M, respectively, induced IF secretion in a dose-dependent manner. However, tetragastrin and histamine did not induce it, even in doses adequate to stimulate acid secretion. A23187, a Ca^{2+} ionophore, and forskolin, an adenylate cyclase activator, strongly stimulated IF secretion in a dose-dependent manner.

DISCUSSION

Since there is no report on IF secretion from rat gastric chief cells, we confirmed the types of chemicals that stimulate IF secretion in rat gastric perfusion. We first attempted to measure IF by CBPCA with ^{14}CN-Cbl. As the rat esophagus was ligated during gastric perfusion, perfusate would not be contaminated by saliva. Therefore,

the substance measured by CBPCA is IF. Gastric secretion is usually influenced by many factors *in vivo*, so that the response of IF secretion might be modified by a chemical. Then, the same dose of a chemical was injected twice at an interval of 2 h. If similar responses are observed to both first and second stimulation by the chemical, we can consider that the stimulant is a true one. As IF and pepsinogen secretion were stimulated by both first and second administrations of carbachol or CCK-8 in rat gastric perfusion, we view these substances to be stimulants of IF. However, no response of IF secretion was seen to the second administration of histamine or tetragastrin, though at these doses they were able to stimulate gastric acid secretion. From these results, it is suggested that histamine and tetragastrin are not stimulants of IF. IF secretion induced by the first stimulation of histamine and tetragastrin might happen to be accompanied by acid secretion.

To confirm the above results, we examined the effects of several kinds of chemicals on IF secretion from isolated gastric chief cells. For this purpose we established a sensitive EIA for the measurement of rat IF, since the sensitivity of CBPCA was too low to measure a small amount of IF because of the low specific activity of ^{14}CN-Cbl. A sufficient amount of purified rat IF for immunization was obtained from the rat gastric mucosa by affinity chromatography. Obtained rat IF was homogeneous and had the same mobility as the standard IF by SDS-PAGE. Cross-reacting bands to anti-rat IF antiserum were seen on the Western blot of stomach homogenate and serum. Based on their molecular weight and the tissues, they might be a dimer of IF or transcobalamin which is a Cbl carrier protein in serum. Using rabbit anti-rat IF antiserum, we were able to measure more than 5×10^{-10} g/well of rat IF with EIA. We confirmed the existence of IF in chief cells by measuring with EIA and a immunohistochemical method. IF content in chief cells was about one fiftieth pepsinogen content, which was estimated in our laboratory. On the basis of the molecular weight of IF being 57 kDa, as estimated by SDS-PAGE, IF content in rat chief cells was 28.9 fmol/10^6 cells. IF content in mammalian gastric mucosa might be almost the same, because it was reported that dispersed human gastric mucosal cells contained 28.4 fmol/10^6 cells of IF.[17] In the submaxillary gland, content of Cbl-binding protein measured by CBPCA is different from that of IF measured by EIA. A large amount of Cbl-binding protein in the submaxillary gland may be R-binder. This result shows anti-IF antiserum did not react to R-binder. Since Cbl-binding protein that has once bound to Cbl does not bind Cbl anymore, the contents measured by EIA were usually higher than those by CBPCA. The reason contents of IF in the liver and kidney are high is not clear. However, it was reported that receptors of IF are present in the kidney.[18]

We examined the effects of several kinds of chemicals on IF secretion from cultured chief cells. Carbachol, CCK-8 and secretin induced IF secretion from cultured rat chief cells, but histamine and tetragastrin did not. A23187 and forskolin also stimulated IF secretion. These results showed that IF secretion resembles pepsinogen secretion in points of the sort of secretagogues and their effective concentrations, height of the responses and the second mediators. We earlier reported that carbachol and CCK-8 induce an intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) increase, and that secretin stimulates a cAMP accumulation in the same kind of chief cells.[15] Therefore we concluded that carbachol and CCK-8 induced IF secretion *via* a $[Ca^{2+}]i$ increase and that secretin induced it *via* a cAMP accumulation. In dispersed gastric mucosal cells from rabbit, guinea pig[19] or human,[17] on the other hand, IF secretion was stimulated by tetragastrin, histamine and carbachol in the same manner as acid secretion was. It is curious that the mechanism of IF secretion depends upon the sort of cells that produce IF.

Several doubts remain, however: Are pepsinogen and IF synthesized and secreted at the same time and do they exist in the same secretory granules? Why is IF secreted from chief cells of only the rat and mouse?

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