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Monoclonal antibody to the gastrin receptor on parietal cells recognizes a 78-kDa protein

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ABSTRACT **Four monoclonal antibodies reactive by immunofluorescence and by flow microfluorimetry with canine and porcine gastric parietal cell membranes were produced by fusion of mouse NS-1 myeloma cells with splenocytes from mice immunized with a population of canine gastric mucosal cells containing 60–70% parietal cells. One of these, an IgM antibody designated 2C1, reacted with the surface membranes of parietal cells by immunofluorescence, flow microfluorimetry, and immunogold electron microscopy; competed with ^{125}I-labeled gastrin for binding to gastric cells; and inhibited by 56% maximal gastrin stimulation of [^{14}C]aminopyrine uptake in parietal cells. The antibody immunoprecipitated ^{125}I-labeled samples of a 78-kDa gastrin-binding protein purified from membrane extracts of porcine gastrin mucosa but did not recognize the same protein labeled covalently with ^{125}I-labeled gastrin-(2–17)-hexadecapeptide. These observations suggest that the previously identified 78-kDa gastrin-binding protein is the gastrin receptor and that the antibody 2C1 is directed against the gastrin binding site of the gastrin receptor.**

Gastrin, a polypeptide hormone belonging to the gastrin/cholecystokinin family of peptides (1) stimulates hydrochloric acid secretion by gastric parietal cells and has a trophic action on the gastrointestinal mucosa (2). Studies of the binding of ^{125}I-labeled synthetic human gastrin have identified gastrin receptors in gastric glands (3), in partially purified preparations of both parietal (4–6) and nonparietal (7) gastric mucosal cells, and in membrane preparations of gastric mucosa (8–10) and antral smooth muscle (11). The receptor has been further characterized as a membrane glycoprotein of 74 kDa by covalent crosslinking of ^{125}I-labeled gastrin-(2–17)-hexadecapeptide to isolated canine parietal cells (12). A gastrin-binding glycoprotein of similar molecular mass (78 kDa) has also been identified by the same technique in membrane extracts of porcine gastric mucosal membranes following purification on wheat germ lectin-Sepharose (13). However, the relationship between these two proteins remains uncertain, since the concentration of gastrin-(1–17)-heptadecapeptide required for 50% inhibition of crosslinking in extracts [0.3 μM (13)] is considerably higher than both the concentration required for 50% inhibition of crosslinking to isolated cells [10 nM (12)] and the K_d (0.5 nM) of the gastrin receptor determined by reversible binding of gastrin-(1–17) to isolated parietal cells (6).

Autoantibodies that inhibit binding of gastrin to the gastrin receptor on rat parietal cells have been found in the sera of some patients with pernicious anemia (14). In the present study, we have produced a monoclonal antibody to the gastrin receptor on canine parietal cells. This antibody binds to the surface membranes of parietal cells as judged by immunofluorescence, competes with ^{125}I-labeled gastrin-(1–17) for binding to parietal cells, inhibits hydrochloric acid secretion assayed by [^{14}C]aminopyrine accumulation, and binds to the 78-kDa gastrin-binding protein purified from membrane extracts of porcine gastric mucosa.

MATERIALS AND METHODS

Production and Screening of Monoclonal Antibodies. Ten-week-old inbred BALB/c mice were immunized by intraperitoneal injection with 10^7 gastric parietal cells (60–70% pure) prepared from canine stomach (14). The mice were given a booster injection 3 weeks later, with 10^7 cells given by the same route. Four days later, the mice were killed, and spleens were removed and teased in Dulbecco's modified Eagle's medium (DMEM) to a single-cell suspension. By use of polyethylene glycol, 10^7 spleen cells were fused with 2×10^7 NS-1 myeloma cells, according to Galfre *et al.* (15). The cell pellet was resuspended in DMEM with 10% fetal bovine serum (FBS) and distributed, in 100-μl aliquots, to four 96-well tissue culture plates (Disposable Products, Adelaide, Australia) containing 3×10^3 inbred BALB/c mouse peritoneal cells per well.

Cultures, incubated at 37°C in an atmosphere of 5% CO_2/95% air, were fed on days 1, 7, and 10 with medium containing hypoxanthine, aminopterin, and thymidine (100 μl per well). After 10–12 days in culture, 50–70% of culture wells contained growing hybrid cells. Hybridoma supernatants were examined for reactivity with canine gastric parietal cells by indirect immunofluorescence as follows. Enriched gastric mucosal cells were incubated with hybridoma supernatant (undiluted) for 30 min at room temperature. Control cells were incubated with phosphate-buffered saline (PBS: 145 mM NaCl/7.5 mM Na_2HPO_4/2.5 mM NaH_2PO_4, pH 7.2) or DMEM/FBS alone. Bound immunoglobulin was detected by treating the cells (30 min, room temperature) with a fluorescein isothiocyanate-labeled sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia) diluted 1:100 in PBS. The cells were viewed with a Leitz Diavert epi-illumination fluorescence microscope. Supernatants from hybrids giving immunofluorescent reactivity with parietal cell surface membranes were selected for further study. These hybrids were grown in hypoxanthine/thymidine-containing medium for 1 week and transferred to DMEM/FBS for cloning by limiting dilution. Aliquots of cloned hybrids were removed from culture, frozen in DMEM/FBS plus 10% dimethyl sulfoxide, and stored in liquid nitrogen. Ascites were obtained from one of the parietal cell-reactive clones, 2C1, by injecting the hybrids into the peritoneum of BALB/c mice. Immunodiffusion analysis showed that 2C1 belonged to the IgM antibody class.

In all subsequent studies, parallel experiments were carried out with an isotype control, 4AII, a monoclonal IgM antibody directed against vimentin intermediate filaments.

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Abbreviation: iBuMeXan, 3-isobutyl-1-methylxanthine.

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Cell Analysis by Flow Microfluorimetry. For measurements of indirect membrane immunofluorescence by flow microfluorimetry, gastric mucosal cell suspensions were incubated with hybridoma supernatant or with DMEM/FBS or PBS controls, using a method described previously (16). Bovine type I DNase (10 μg/ml; Sigma) was added to the cell suspensions to prevent cell clumping. Cell analysis and sorting were carried out on a fluorescence-activated cell sorter (FACS II; Becton Dickinson) equipped with an argon laser operated at 488 nm and 400 mW for fluorescence excitation. Fluorescence was detected with an S-11 photomultiplier tube (EMI Electronics, Middlesex, England) at 550 V with a 520-nm barrier filter.

Immunogold Electron Microscopy. Parietal cell-enriched gastric mucosal cells (10^6 per ml) were fixed in 2.5% glutaraldehyde (20 min, room temperature). The cells were washed three times by centrifugation (250 × *g*, 5 min) in PBS, resuspended in 0.1 M NH_4Cl (15 min, room temperature), washed three times (250 × *g*, 5 min) in PBS, and incubated (40 min, 37°C) with monoclonal antibody 2C1. Control preparations were incubated with an isotype control and in culture medium. The cells were washed and incubated (30 min, 37°C) with sheep anti-mouse immunoglobulin (0.2 mg/ml; Silenus). Bound immunoglobulin was traced by incubation (30 min, room temperature) with protein A bound to colloidal gold (17 nm; OD_{520} = 0.460), prepared from protein A (Pharmacia) and chloroauric acid (Sigma) according to a published method (17). The cells were washed, post-fixed in 1% OsO_4, dehydrated in alcohol, processed for embedding in Epon/Araldite, and stained with uranyl acetate and Reynold's lead citrate. The sections were examined with a JOEL 100S electron microscope.

Gastrin Binding Assay. The assay has been described (14). In brief, gastric mucosal cell preparations (3×10^6 cells) were preincubated (30 min, room temperature) with or without 100 μl of hybridoma antibody in DMEM. The cells were washed twice by centrifugation (250 × *g*, 2 min) in DMEM before exposure to gastrin. The cells (3×10^6 per well) were suspended in 90 μl of culture medium containing 10% FBS, together with 10 μl of 10 mM Hepes buffer (pH 7.2) containing 1% bovine serum albumin (buffer A), and were incubated (30 min, 37°C) in flat-bottomed Terasaki plates with or without 5 μM human [Leu^{15}]gastrin-(1–17) (Research Plus, Bayonne, NJ) in a humidified atmosphere containing 5% CO_2 in air. Aliquots (10 μl) of ^{125}I-labeled human [Leu^{15}]gastrin-(1–17) (specific activity 990 μCi/μg; New England Nuclear; 1 Ci = 37 GBq) at final concentrations ranging from 1 pM to 10 nM in buffer A were added to triplicate wells. After incubation for 45 min at 37°C, the plates were placed on ice. The cells were recovered on Whatman GF/D filters (presoaked overnight at 4°C in 10% bovine serum albumin) with an Amicon 12-port vacuum manifold. The filters were washed twice with 1 ml of buffer A and dried at 50°C for 20 min; bound ^{125}I-labeled gastrin was measured with a gamma counter with an efficiency of 80% (Packard). Specific binding was calculated as the difference between counts bound in the absence and in the presence of 5 μM [Leu^{15}]gastrin-(1–17). Inhibition of gastrin binding was expressed as the percentage reduction of specific binding caused by the hybridoma antibody. Experiments were also carried out with hybridoma antibodies that had been absorbed with gastric, liver, or kidney cells. Absorption was carried out by overnight incubation at 4°C of 100 μl of antibody with 10^7 cells.

[^{14}C]Aminopyrine Accumulation Bioassay. The [^{14}C]-aminopyrine bioassay (an index of gastric acid secretory activity) has been described in detail (18–20). Aliquots (1 ml) of parietal cell-enriched cell suspensions containing 3×10^6 cells per ml in DMEM/10% FBS were placed in 1.5-ml conical polypropylene tubes and incubated (20 min, room temperature) with hybridoma supernatant (undiluted). The cells were washed twice in serum-supplemented DMEM by centrifugation (250 × *g*, 5 min) and resuspended in 1 ml of the same medium containing 3 μM [*dimethylamino*-^{14}C]aminopyrine (specific activity 114 Ci/mmol; Amersham), 10 μM 3-isobutyl-1-methylxanthine (iBuMeXan; Sigma), 0.5 mM dithiothreitol (Sigma), and synthetic [Leu^{15}]gastrin-(1–17) (Fluka) at final concentrations ranging from 0.1 nM to 1 μM.

The tubes were sealed and incubated by submersion in a 37°C water bath. The samples were mixed by horizontal shaking (110 cycles per min, 45 min). Duplicate 0.5-ml aliquots of each sample were washed in 1 ml of Hank's balanced salts solution by centrifugation (1000 × *g*, 1 min). The supernatants were aspirated, and the cell pellets were digested in 100 μl of Protosol (New England Nuclear) for 30 min at 50°C before addition of aqueous scintillation fluid and measurement of radioactivity with a liquid scintillation counter (Packard). Included in each run were a positive control (cells exposed to [^{14}C]aminopyrine, iBuMeXan, gastrin, and dithiothreitol without preincubation with the hybridoma supernatant) and a negative control (cells exposed to [^{14}C]aminopyrine, iBuMeXan, and dithiothreitol). Control experiments were also carried out with other, unrelated hybridoma supernatants and with histamine or carbachol stimulation of [^{14}C]aminopyrine accumulation by parietal cells. The accumulation ratio was calculated as cpm per parietal cell volume divided by cpm in the medium. The specific accumulation ratio was calculated as the difference between the ratio in the presence and in the absence of gastrin, histamine, or carbachol. Inhibition of accumulation ratio was expressed as the percentage reduction in the specific accumulation ratio caused by the hybridoma antibody. The statistical significance of results was assessed by Student's *t* test.

Preparation of ^{125}I-Labeled Gastrin-Binding Protein. The gastrin-binding protein was partially purified from Triton X-100 extracts of membranes isolated from the mucosa of porcine gastric corpus as described (13), except that concanavalin A-Sepharose was used in place of wheat germ lectin-Sepharose, before chromatography on DEAE-Sepharose (Pharmacia). The content of gastrin receptor in samples following DEAE-Sepharose chromatography was ≈10%, as assessed by Coomassie blue staining after $NaDodSO_4$/polyacrylamide gel electrophoresis (21). The preparations were iodinated by the chloramine-T method (22) and separated from free iodide by chromatography on a column (15 × 1.0 cm) of Bio-Gel P-6 (Bio-Rad). Alternatively, the preparations were treated with disuccinimidyl suberate and ^{125}I-labeled [Nle^{15}]gastrin-(2–17) as described (13) to form the covalent ^{125}I-labeled [Nle^{15}]gastrin-(2–17)–gastrin-binding protein complex.

Immunoprecipitation. An ^{125}I-labeled preparation of gastrin-binding protein (20 μl, 30,000 cpm) was incubated with 110 μl of hybridoma supernatant for 1 hr at 4°C. The immune complex was incubated for 30 min at 4°C with 50 μl of affinity-purified sheep anti-mouse gamma globulin coupled to Sepharose 4B. The immunoprecipitate was centrifuged (10,600 × *g*, 30 sec), washed five times with PBS, resuspended in 50 μl of $NaDodSO_4$ sample buffer under reducing or nonreducing conditions, and heated at 100°C for 5 min. After centrifugation (10,600 × *g*, 30 sec), the supernatants were subjected to $NaDodSO_4$/polyacrylamide slab gel electrophoresis. The gel was dried on gel baking paper (Bio-Rad) and radioactivity was detected by autoradiography using an intensifying screen (Dupont).

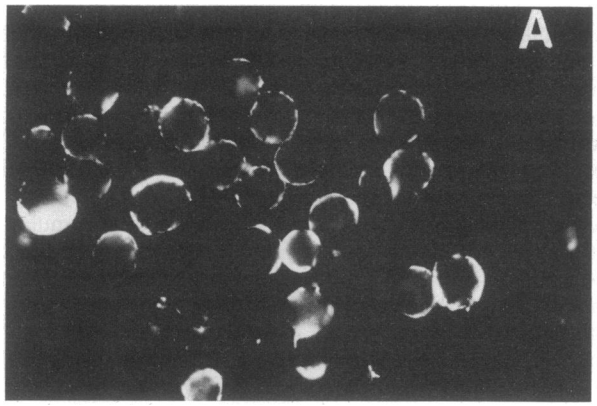
RESULTS

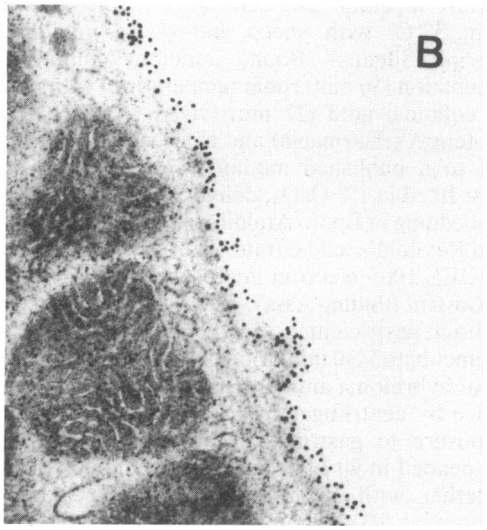
Immunofluorescence, Electron Microscopy, and Flow Microfluorimetry. Eleven hundred thirty-eight hybridomas were isolated from mice immunized with canine parietal cells.

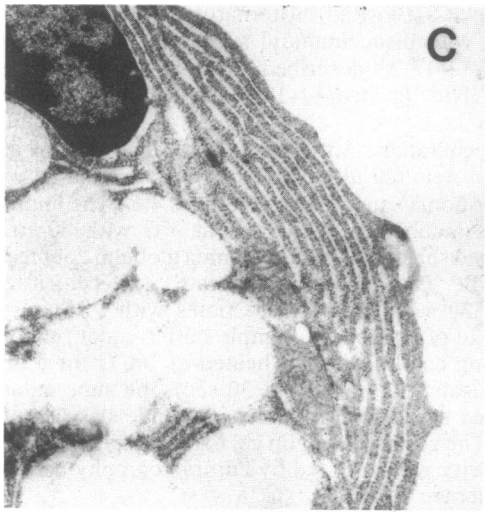
Reaction between four (0.35%) of the supernatants from these hybridomas and the surface membranes of canine parietal cells was detected by indirect immunofluorescence (Fig. 1*A*). The pattern of immunofluorescent staining was similar to that seen with human autoantibody to parietal cells from patients with pernicious anemia (23). Canine chief cells (identified by phase-contrast microscopy), lymphocytes, and kidney or liver cell suspensions did not stain. Immunogold electron microscopy confirmed that the antibody 2C1 labeled the surface membranes of parietal cells, readily identified by the presence of abundant mitochondria (Fig. 1*B*), but did not label the surface membranes of chief cells (Fig. 1*C*). In flow microfluorimetry, the monoclonal antibodies showed significant cell surface staining when the instrument was "gated" for parietal cells on the basis of size (16). The percentage of cells with a cell surface fluorescence intensity registering 50 channel units was 37.3 for antibody 2C1 (Fig. 1*D*), 16.7 for 1C2, 20.6 for 1B1, and 18.4 for 1A5. With porcine parietal cells, cell surface fluorescence intensity registering 50 channel units was observed for 74.1% of the cells incubated with 2C1, but the remaining three monoclonal antibodies did not give readings greater than the negative control.

Gastrin Binding Assay. Antibody 2C1 inhibited specific binding of ^{125}I-labeled gastrin to parietal cells by 50.6 ± 1.9% (two separate experiments) at 10 nM ^{125}I-labeled gastrin (Fig. 2*A*). Inhibition was seen over the full range of ^{125}I-labeled-gastrin concentrations tested and with concentrations of the antibody greater than 10 μg/ml (Fig. 2*B*). The inhibition was abolished by prior absorption of the antibody with parietal cell-enriched gastric mucosal cells but not by absorption with canine liver or kidney cells (data not shown). The other three monoclonal antibodies giving immunofluorescence staining of the surface membranes of parietal cells did not inhibit binding of ^{125}I-labeled gastrin to parietal cells.

[^{14}C]Aminopyrine Accumulation Assay. [^{14}C]Aminopyrine uptake is an accurate index of gastric acid secretory activity of isolated canine parietal cells (18). The maximal response to gastrin (3.8 times basal) and ED_{50} for gastrin (2 nM) were both similar to the values reported by Soll (18). Antibody 2C1 inhibited by 56.0 ± 11.0% (three separate experiments) the







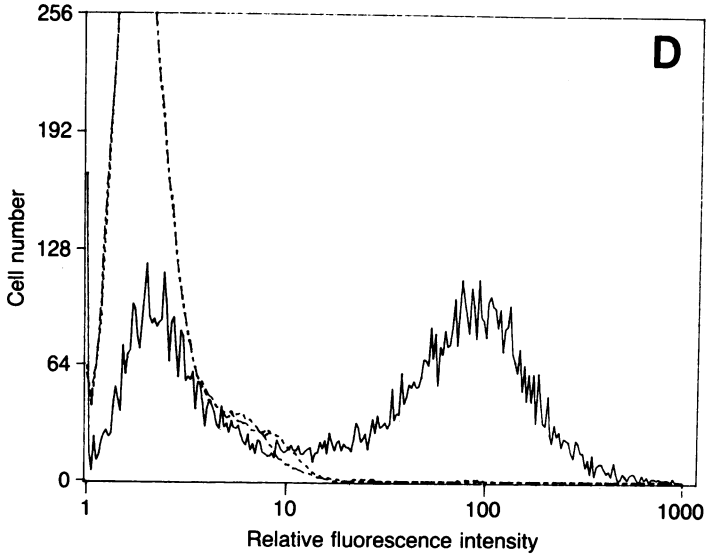


FIG. 1. Reaction of monoclonal antibody 2C1 with surface membranes of canine gastric parietal cells, as detected by immunofluorescence microscopy (*A*; ×850), immunogold electron microscopy (*B*; ×42,500), and flow microfluorimetry [*D*; solid line, 2C1; broken line, isotype control (IgM antibody to vimentin intermediate filaments)]. No reaction was detected between antibody 2C1 and canine chief cells by immunogold electron microscopy (*C*).

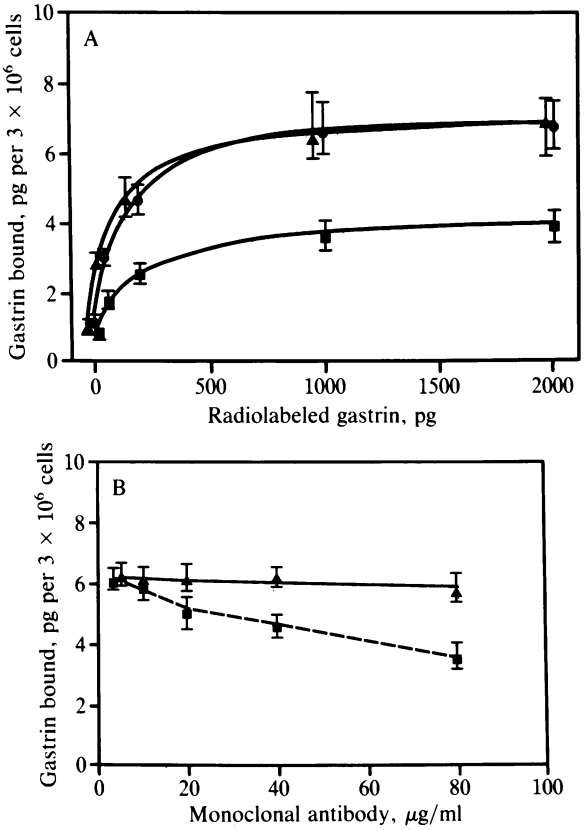


FIG. 2. Inhibition of binding of ^{125}I-labeled gastrin to gastric parietal cells by 2C1. Gastric mucosal cell preparations (3×10^6 cells, 60–70% parietal cells) were preincubated with antibody 2C1 (■), with an isotype control (●), or without antibody (▲) for 30 min at room temperature. After removal of excess antibody by washing, binding of ^{125}I-labeled [Leu^{15}]gastrin-(1–17) was measured, using various concentrations of radiolabeled gastrin (*A*) or 1 nM labeled gastrin in the presence of various concentrations of monoclonal antibody (*B*). Points represent the means of triplicate samples, corrected for nonspecific binding [measured in the presence of 5 μM unlabeled gastrin-(1–17)]. Inhibition by antibody 2C1 was statistically significant ($P < 0.01$) at all gastrin concentrations.

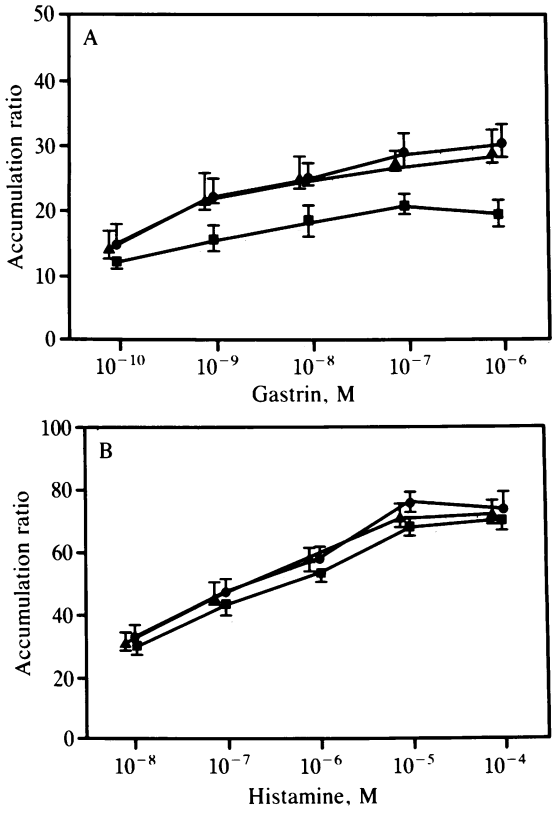


FIG. 3. Inhibition of gastrin-stimulated [^{14}C]aminopyrine uptake by 2C1. Gastric mucosal cell preparations (3×10^6 cells, 60–70% parietal cells) were preincubated with antibody 2C1 (■), with an isotype control (▲), or without antibody (●) for 10 min at room temperature. The acid-secretory activity of the cells after stimulation by gastrin (*A*) or histamine (*B*) in the presence of 10 μM iBuMeXan and 0.5 mM dithiothreitol was measured by [^{14}C]aminopyrine uptake (18, 19) as described in *Materials and Methods*. Points represent the mean of triplicate samples. Inhibition by antibody 2C1 was statistically significant ($P < 0.01$) at all gastrin concentrations.

maximal gastrin stimulation of [^{14}C]aminopyrine accumulation by parietal cells (Fig. 3*A*) but did not inhibit maximal histamine (Fig. 3*B*) or carbachol (data not shown) stimulation of [^{14}C]aminopyrine accumulation. The other three monoclonal antibodies reactive by immunofluorescence with the surface membranes of parietal cells did not inhibit gastrin, histamine, or carbachol stimulation of [^{14}C]aminopyrine accumulation. None of the monoclonal antibodies stimulated [^{14}C]aminopyrine uptake by parietal cells (data not shown).

Immunoprecipitation. Antibody 2C1 immunoprecipitated a 78-kDa protein from an ^{125}I-labeled preparation of membrane proteins purified from a Triton X-100 extract of porcine gastric mucosal membranes by chromatography on concanavalin A-Sepharose and DEAE-Sepharose (13) (Fig. 4). ^{125}I-labeled gastrin-(2–17) crosslinked to the same gastric mucosal preparation by disuccinimidyl suberate also identified a 78-kDa protein (13). However, antibody 2C1 did not immunoprecipitate the crosslinked gastrin–78-kDa-protein complex (Fig. 4). The other three parietal cell surface-reactive antibodies did not immunoprecipitate either the ^{125}I-labeled 78-kDa protein or the crosslinked gastrin–78-kDa-protein complex. Attempts to confirm the immunoprecipitation data by immunoblotting of gastric mucosal membrane proteins with the antibody 2C1 were uniformly negative, implying that the antigenic determinant is denatured during $NaDodSO_4$/polyacrylamide gel electrophoresis.

DISCUSSION

Binding of the monoclonal antibody 2C1 to the surface of canine and porcine parietal cells was detected by immunofluorescence and by flow microfluorimetry. Antibody 2C1 did not react with canine gastric chief cells or with canine lymphocytes, kidney, or liver cells. These observations were confirmed by ultrastructural studies showing gold labeling of the membrane of parietal cells, but not of chief cells, by 2C1. The results suggested that the antigen recognized by the monoclonal antibody 2C1 was present on parietal cells only and, furthermore, was restricted to the cell surface. The antibody also inhibited, in a dose-dependent manner, binding of ^{125}I-labeled gastrin-(1–17) to parietal cells. Inhibition of gastrin binding was not observed after absorption of the antibody with parietal cell-enriched gastric mucosal cells but was observed after absorption with canine liver or kidney cells. These observations suggested that monoclonal antibody 2C1 recognized the gastrin receptor on parietal cells and competed with gastrin for binding to the gastrin receptor. The results of these studies were supported by the observation that antibody 2C1 inhibited gastrin-stimulated but not histamine- or carbachol-stimulated accumulation of [^{14}C]aminopyrine by parietal cells.

The hypothesis that 2C1 recognizes the gastrin binding site of the gastrin receptor could be further tested by examining the effect of increasing concentrations of gastrin on the inhibitory effects of the antibody. If the inhibition is truly competitive, and the affinities of the receptor for gastrin and the antibody are similar, then it should be possible to

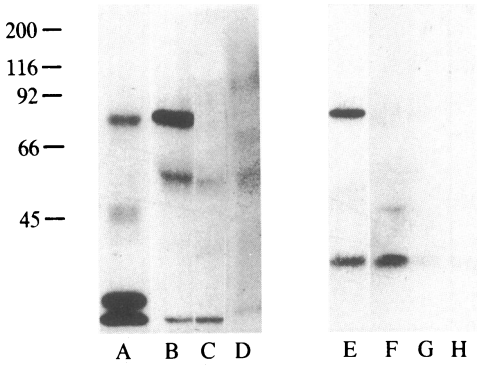


FIG. 4. Immunoprecipitation of 78-kDa gastrin-binding protein. The 78-kDa gastrin binding protein was purified from Triton X-100 extracts of porcine gastric mucosal membranes by chromatography on concanavalin A-Sepharose and DEAE-Sepharose (13) and was directly iodinated with chloramine T (lanes A–D, 2-day autoradiographic exposure) or covalently crosslinked to ^{125}I-labeled [Nle^{15}]gastrin-(2–17) with disuccinimidyl suberate (13) (lanes E–H, 7-day exposure). Samples of the starting materials (lanes A and E) were immunoprecipitated with 2C1 (lanes B and F), with an isotype control (lanes C and G), or with medium alone (lanes D and H). The immunoprecipitates were solubilized and subjected to electrophoresis in $NaDodSO_4$/polyacrylamide gels. Labeled proteins were visualized by autoradiography. Markers at left show positions and molecular mass (kDa) of standard proteins run in parallel.

overcome the inhibitory effect of the antibody in either the gastrin binding or aminopyrine uptake assays simply by increasing the concentration of gastrin. Our current data indicate that the antibody still inhibits aminopyrine uptake in the presence of 1 μM gastrin (Fig. 3*A*) and hence imply that the affinity of the receptor for antibody is considerably greater than the affinity of the receptor for gastrin [K_d = 0.5 nM (18)].

Antibody 2C1 immunoprecipitated a 78-kDa protein from ^{125}I-labeled preparations of membrane proteins from porcine gastric mucosa. The protein has been previously identified as a possible solubilized form of the gastrin receptor by covalent crosslinking to ^{125}I-labeled gastrin-(2–17) (13). However, although the size of the 78-kDa protein was very similar to the size (74 kDa) of the gastrin receptor identified by the same technique on canine parietal cells (12), the affinity of the solubilized receptor for gastrin was between 30 and 200 times lower than that of the membrane-bound receptor, depending on the detergent used for solubilization (13). Immunoprecipitation by the 2C1 monoclonal antibody, which blocked the binding of ^{125}I-labeled gastrin to isolated parietal cells, confirmed that the 78-kDa protein is indeed the gastrin receptor and hence implied that the affinity of the receptor for gastrin is decreased upon detergent extraction. Failure of 2C1 to immunoprecipitate the crosslinked gastrin receptor complex further suggested that the antibody recognizes the gastrin binding site on the receptor. This suggestion is consistent with the competition observed between the antibody and ^{125}I-labeled gastrin for binding to parietal cells. Antibody 2C1 should be a useful tool for studies of the distribution, structure, and function of the gastrin receptor.

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