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THE PRESENCE OF A 33-40 KDa GASTRIN BINDING PROTEIN ON HUMAN AND MOUSE COLON CANCER

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SUMMARY: Human and mouse colon cancers have specific binding sites for gastrin and demonstrate a trophic response to gastrin. In the present study we used radiolabeled gastrin (2-17), to determine the molecular weight of gastrin binding proteins (receptors) on mouse and human colon cancers, by cross-linking methods. Crude membrane aliquots prepared from the tumors were radiolabeled with [^{125}I]gastrin (2-17) ± 1000 fold excess of unlabeled gastrin and cross-linked with 1 mM disuccinimidyl suberate. The cross-linked radiolabeled binding protein complexes were solubilized and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The autoradiographs of the gels demonstrated the presence of a predominant band of ~33-40 KDa gastrin binding protein, that was specific for gastrin analogs. Our present findings thus indicate that specific gastrin binding proteins/gastrin receptors on colon cancers are primarily present as one band with a molecular mass of ~33-40 KDa and are specific for gastrin-like peptides.

A gastrointestinal hormone, gastrin, is known to be a trophic agent for a number of gastrointestinal mucosal cells (1) and also stimulates the growth of colon cancer cells, both in vivo (reviewed in 2) and in vitro (3-7). We recently reported the presence of gastrin receptors (GR) on human colon cancers (8). GR are also present on a mouse colon cancer (MC-26) cell line (9). Significant trophic effects of gastrin on the MC-26 cells in vivo (9) and in vitro (10) were reported, which appeared to be mediated by the high-affinity gastrin receptors (GR) present on the MC-26 cells (10-12). The above studies with human and mouse colonic cancers, thus indicated a possible important role of gastrin and GR in the growth of some colon cancers.

The molecular mass of GR on canine parietal cells was recently reported to be 74 KDa (13). Cross-linking studies of GR on porcine fundic mucosal cells, on the

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other hand, indicated the presence of at least four molecular bands of gastrin binding proteins with a molecular mass of 34, 51, 65, 78 and >200 KDa; the 78 KDa protein being the predominant molecular form of the gastrin binding proteins on the porcine fundic mucosa (14). We similarly observed the presence of at least four molecular forms of gastrin binding proteins on the guinea pig and mouse fundic mucosal cells (15,16), with molecular mass ranging from 33-72 KDa. Our preliminary studies with molecular weight determination of GR on MC-26 cells (17), however, indicated that only one 33-40 KDa gastrin binding protein was present on the MC-26 cells. In the present studies we have confirmed these findings with both mouse and human colon cancers, indicating that colon cancers unlike the normal gastrointestinal mucosal cells, have only one molecular species of gastrin binding proteins, which is apparently the smallest of the various forms reported to be present on the normal gastrointestinal mucosal cells.

MATERIALS AND METHODS

Transplantation of Mouse Colon Cancer Cells in Mice: A transplantable mouse colon cancer, CT26, was obtained from Mason Research Laboratory (Worcester, MA); from which a tissue culture cell line, MC-26, was established in our laboratory. MC-26 cells, 24-48 h postplating were suspended in HBSS as given above, and single cell suspensions were inoculated into male Balb/c mice (20-25 g, Harlan-Sprague Dawley, Houston, TX). Cancer cells (0.5×10^6) were injected s.c. in the flank, and 15-21 days post-inoculation, the tumors were harvested from the mice. Mice were decapitated, and tumors quickly removed in ice cold 0.9% NaCl solution and washed extensively to remove extraneous contamination. Tumors had well defined margins and could be dissected free from surrounding normal tissues without difficulty. All other steps were carried out at 4^0C unless otherwise stated. Tumors were washed in Buffer A (10 mM Tris, 146 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.25 M sucrose, pH 7.4) containing [0.1% bovine serum albumin (BSA, Fraction V), 0.1% soybean trypsin inhibitor and 0.1% bacitracin (BSB), (Sigma Chemical Co., St. Louis, MO)]. Tumors thus collected were snap frozen in liquid nitrogen and stored at -70^0C in an ultra deep freezer for a maximum of 7-15 days.

Collection of Human Colon Cancers: Specimens of primary colon carcinomas were obtained at the time of surgical resection from patients treated at the University of Texas Medical Branch Hospitals. All specimens were examined by the Surgical Pathology Service. Immediately upon removal of the surgical specimens, portions of viable cancer were excised for the measurement of gastrin receptors and other studies.

Preparation of Tumor Membranes: Frozen tumors were pulverized with a Thermovac autopulverizer cooled in liquid nitrogen. The resulting powder was homogenized in five volumes of Buffer A + BSB, using a precooled Polytron homogenizer (PG-10-ST, Brinkman Industries, Westbury, NY). The homogenate was filtered through a double layer of cheesecloth, presoaked in ice-cold Buffer A and subjected to 200 x g, 10 min centrifugation to remove any cell debris. For the preparation of total crude membrane fractions, the 200 x g 10 min supernatant was subjected to 30,000 x g, 45 min centrifugation in a fixed angle SM-24 rotor. Pellets were washed once again with Buffer A + BSB and repelleted at the same speed. The pellet thus obtained was resuspended in the binding Buffer B (25 mM 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid, 5 mM $MgCl_2$, pH 7.4) + BSB, homogenized manually with a glass teflon homogenizer, and processed for measurement of protein.

Gastrin Binding to Membranes: Synthetic human gastrin-(1-17)-I and human gastrin (2-17)-I (Research Plus, Bayonne, NJ) were iodinated with Iodo-Gen (Pierce

Laboratory, Rockford, IL) as described previously (18). The radiolabeled peptides were purified on an ion exchange column, as described previously (18), and specific activity calculated to be ~1-1.5k dpm/fmole. For the purposes of binding affinity measurements, aliquots of membrane suspension were incubated with increasing concentration (0.01-10.0 nM) of [^{125}I]gastrin ± 1000 fold excess gastrin at 30^{0}C for 30 min and the binding affinity determined from a Scatchard plot (19) of specific binding data as described previously (18). For the purpose of relative binding affinity measurement, aliquots of membrane suspension were incubated with [^{125}I]gastrin (1 nM) ± increasing concentrations of various peptides for 30 min at 30^{0}C and the relative binding affinity (RBA) of competing peptides determined from the log dose-percent inhibition curve as described previously (18). For the purpose of cross-linking studies, aliquots of membrane suspension in Buffer B + BSB, containing 150 μg protein, were incubated with 10 nM [^{125}I]gastrin (2-17) ± 1000x excess of either the homologous or other radioinert peptides (Peninsula Laboratories, Belmont, CA), for 15 min at 30^{0}C. At the end of the reaction, the binding assay tubes were chilled on ice and excess ice cold Buffer B + BSB added. The peptide hormone bound to the membrane was quickly separated from excess unbound hormone by centrifugation in a microcentrifuge (Model 235B, Fisher Scientific, Houston, TX) at 4^{0}C and the membrane pellet washed two times with the binding buffer in the presence (affinity measurement studies) or absence (cross-linking studies) of BSA. The washed samples from the affinity measurement experiments were counted for [^{125}I] in a gamma counter (Beckman Minaxγ 5000 series, Houston, TX) with ~75% counting efficiency. Membranes labeled with [^{125}I]gastrin (2-17) were then subjected to cross-linking as given below.

Molecular Weight Determination of GR by Cross-Linking Studies: Aliquots (1 ml) of the labeled membranes were incubated with 1 mM disuccinimidyl suberate (DSS, Sigma) for 15 min at 22^{0}C. DSS was dissolved in dimethyl sulfoxide (DMSO), immediately prior to use and added to the substrate to give a final concentration of 1% of DMSO in the binding buffer. The reaction was terminated by rapid centrifugation. The membranes were washed with excess binding buffer, devoid of BSA. The washed membrane pellets in the reaction tubes were solubilized by boiling at 100^{0}C for 3-5 min in 0.2 M Tris HCl buffer, pH 6.8, containing 6% sodium dodecyl sulfate (SDS w/v), 2 mM EDTA, 10% glycerol (v/v) in the presence or absence of 4% β-mercaptoethanol (v/v). The supernatant was then subjected to SDS polyacrylamide gel electrophoresis according to the method of Laemmli (20) using 9.5% acrylamide in the separating gel and 4% acrylamide in the stacking gel. The gels were stained, destained, dried and exposed to Kodak XAR-5 film for 7-14 days at -70^{0}C. The autoradiograms were scanned using a double-beam densitometer and the area under the peaks measured with a Hewlett-Packard digitizer.

Protein Measurements: Protein was measured by the methods of Lowry et al (21).

RESULTS

Human colon cancer specimens from patients, determined to be positive for high-affinity gastrin binding sites (gastrin receptors, GR) (Fig 1), and MC-26 tumors (known to be highly positive for gastrin binding [9,12]), were used for characterizing the molecular weight of specific gastrin binding proteins by cross-linking methods outlined under the methods section. The autoradiographic profiles of the specifically bound [^{125}I]gastrin (2-17) to GR from human colon tumor and MC-26 tumor membranes is shown in Figures 2 and 3, respectively. One major band, radiolabeled specifically with gastrin, was evident in samples from both the human (Fig 2) and mouse colon (Fig 3) tumor membranes, with molecular mass of ~33-40 KDa. A minor band of gastrin binding proteins, that was apparently specfific for binding gastrin, was also observed at the origin of the gel (Figs 2-4), indicatin

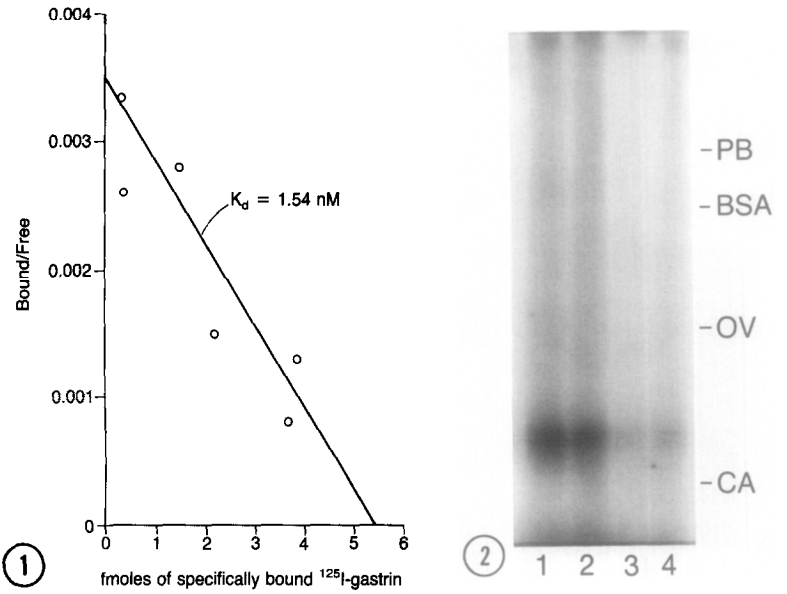

Figure 1. Scatchard plot of specific binding of [^{125}I]gastrin (1-17) to human colon cancer membranes from a patient.

Figure 2. Autoradiographic profile of [^{125}I]gastrin (2-17) binding to GR on human colonic tumor membranes. Membranes were labeled with [^{125}I]gastrin (2-17) ± 1000 fold excess of gastrin and cross-linked with 1 mM DSS as given under Methods. Solubilized proteins from the membranes, were subjected to SDS-PAGE under reducing conditions and analyzed autoradiographically. The arrows indicate the position of the standard molecular weight marker proteins (PB = phospholipase B, BSA = bovine serum albumin, OV = ovalbumin, CA = carbonic anhydrase, SBTI = soya bean trypsin inhibitor, LYSO = lysozyme). Data from two separate patients is presented as representative of data from a total of eight patients analyzed. Lanes 1 and 2 = total binding of [^{125}I]gastrin (2-17) to tumor membranes from two separate patients, A and B. Lanes 3 and 4 = non-specific binding of [^{125}I]gastrin (2-17) in the presence of 1000x excess unlabeled gastrin to tumor membranes from patients A and B.

a possible presence of a very high molecular weight gastrin binding protein on colonic cancers.

The specific binding of gastrin to the 33-40 KDa binding protein on MC-26 tumors was displaced in a dose-dependent manner by the homologous peptide (Fig 4), but was not displaced to any significant level by increasing doses of unrelated peptides (vasoactive intestinal peptide, bombesin and luteinizing hormone releasing hormone). Cholecystokinin-octapeptide (CCK), a closely related peptide, demonstrated negligible binding affinity for the ~33 KDa gastrin binding protein on the MC-26 tumor membranes, and inhibited gastrin binding only at very high concentrations (50 μM or more) (Fig 4 and Fig 5).

A dose-dependent inhibition of the binding of [^{125}I]gastrin to the human colonic tumor membranes by the homologous peptide was also observed (Fig 5). CCK demonstrated a significantly lower binding affinity for the gastrin binding

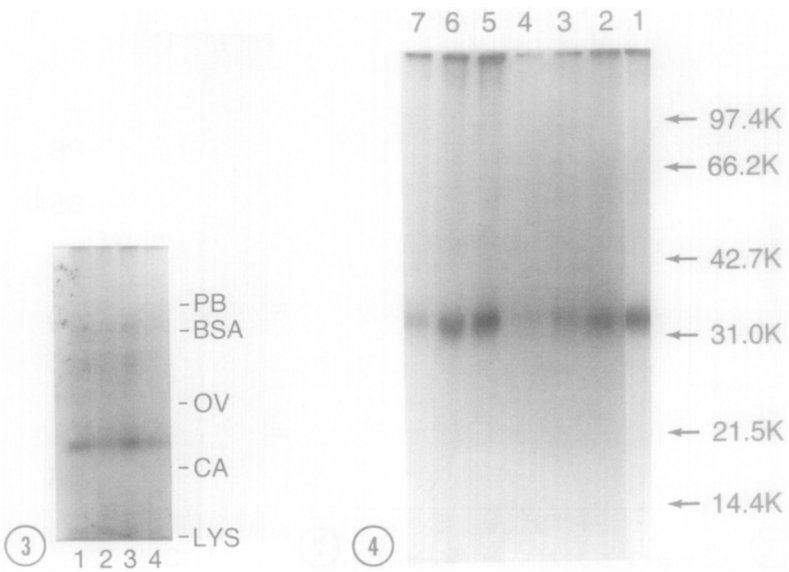

Figure 3. Autoradiographic profile of [^{125}I]gastrin (2-17) binding to GR on MC-26 tumor membranes. Membranes were labeled with [^{125}I]gastrin (2-17) ± 1000-fold excess of gastrin and analyzed as given under Figure 2. The arrows indicate the position of the standard molecular weight marker proteins. Data from two separate mouse tumors is presented as representative data of several similar studies. Lanes 1 and 3 = total binding of [^{125}I]gastrin (2-17) to two separate tumors A and B, respectively. Lanes 2 and 4 = non-specific binding of [^{125}I]gastrin (2-17) to the two tumors A and B, respectively.

Figure 4. Competitive displacement of [^{125}I]gastrin (2-17), cross-linked to the 33-40 KDa gastrin binding proteins on MC-26 tumor membranes, by increasing concentrations of gastrin and CCK. Membranes derived from MC-26 tumors, were labeled and cross-linked to 1 nM [^{125}I]gastrin (2-17) ± increasing concentrations of either gastrin or CCK. The solubilized proteins from the thus treated membranes were subjected to SDS-PAGE under reducing conditions and analyzed autoradiographically. The peptide concentration in lanes 1-7 was as follows: Lane 1 = 0 (total binding); Lanes 2-4 = gastrin; Lanes 5-7 = cholecystokinin; Lanes 2, 5 = 1.0 μM; Lanes 3, 6 = 10.0 μM; Lanes 4, 7 = 50.0 μM.

proteins compared to gastrin, on both human colonic and MC-26 tumor membranes (Fig 5). From the log-dose inhibition of specific binding of [^{125}I]gastrin to tumor membranes (Fig 5), the relative binding affinity of CCK for the gastrin binding sites on colon cancers was calculated to be <2% compared to 100% of gastrin.

DISCUSSION

In the present study, we have demonstrated the presence of ~33-40 KDa gastrin binding proteins on a mouse colon cancer cell line (MC-26 cells) and several freshly resected human colon cancers, that were specific for binding gastrin-like peptides. A closely related peptide, CCK, demonstrated significantly lower binding affinity for the gastrin binding sites (gastrin receptors, GR) on both MC-26 tumors and human colonic cancers, compared to gastrin. In the case of the normal target cells (such as the parietal or the fundic mucosal cells), it has been

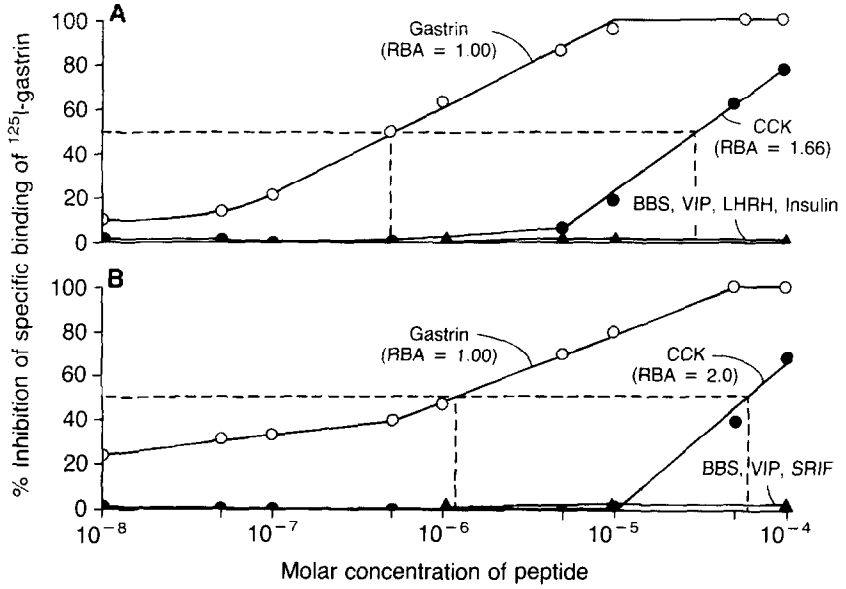

Figure 5. Log-dose inhibition of specific binding of [^{125}I]gastrin (1-17) to human (A) and MC-26 (B) colon cancer tumor membranes by various peptides. The relative binding affinity (RBA) of CCK was calculated from the linear part of the curve as published previously (18), and RBA values thus determined are indicated. The RBA of gastrin was arbitrarily assigned a value of 1.0 (=100%).

reported that CCK binds the specific gastrin binding proteins (GR) equally well or with only slightly lower affinity (22,23). These studies thus indicate that the GR molecule on colon cancer cells is probably different from that present on the normal target cells, at least in terms of relative binding affinity for a closely related peptide, CCK.

Another important difference that became apparent from the present studies, is that while in the colon cancer cells a 33-40 KDa gastrin binding protein was the only molecular species predominantly present, in the normal gastric mucosal cells, one to four molecular species of gastrin binding proteins have been described, which range in molecular weight from 34 to >200 KDa (13-16,24). The difference in the apparent molecular weight of the gastrin binding proteins may thus represent either a species difference and/or an actual difference in the molecular weight of the gastrin binding proteins in normal fundic mucosal cells vs. colonic cancer cells.

The observed differences in the size of the gastrin binding proteins in normal vs. cancer cells, however, could be due to an increased proteolytic cleavage of the binding proteins in the cancer cells, such that a smaller protein (that retains the binding sites), results. In the case of BBS receptors on Swiss 3T3 cells, a 45 KDa binding protein was observed that apparently represented the binding core

of a larger receptor protein (25). In the present studies, however, the 33-40 KDa gastrin binding protein persisted as the dominant molecular form in samples treated with or without various protease inhibitors. The 33-40 KDa binding protein may thus represent the receptor protein present in situ on the cancer cells, and not a proteolytic cleavage product. We did not observe any difference in the size of the binding proteins separated under reducing or non-reducing conditions suggesting that the binding proteins did not represent subunits linked by disulfide bonds.

Thus our data indicates that of the many different molecular species of GR reported to be present on the normal fundic mucosal cells, only one form is present on the colonic cancer cells, which besides being smaller in molecular mass, also demonstrates an altered binding affinity for CCK, compared to the gastrin binding proteins present on the normal gastric mucosal cells. In the case of CCK-receptors, similarly, different forms and types of receptors have been reported to be present on different target cells (26,27), raising the possibility that different target cells may process the binding proteins (receptors) differently.

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REFERENCES

1. Johnson, L.R. (1982) Scand. J. Gastroent. 74:89-92.
2. Townsend, C.M., Jr., Singh, P., and Thompson, J.C. (1986) Gastroenterology 91, 1002-1006.
3. Sirinek, K.R., Levine, B.A., and Moyer, M. (1985) Am. J. Surg. 149, 35-39.
4. Moyer, M.P, Armstrong, A., Aust, J.B., Levine, B.A., and Sirinek, K.R. (1986) Arch. Surg. 121, 285-288.
5. Kusyk, C.J., McNiel, N.O., and Johnson, L.R. (1986) Am. J. Physiol. 251, G597-G601.
6. Watson, S.A., Durrant, L.G., and Morris, D.L. (1988) Br. J. Surg., 75, 342-345.
7. Hoosein, N.M., Kiener, P.A., Curry, R.C., Rovati, L.C., McGilbra, D.K., and Brattain, M.G. (1988) Cancer Res. 48, 7179-7183.
8. Upp, J.R., Jr., Singh, P., Townsend, C.M., Jr., Thompson, J.C. (1989) Cancer Res 49, 488-492.
9. Singh, P., Walker, J.P., Townsend, C.M., Jr., and Thompson, J.C. (1986) Cancer Res. 46, 1612-1616.
10. Guo, Y.-S., Jin, G.-F., Townsend, Jr., C.M., Thompson J.C., and Singh, P. (1988) Pro. Chin. Acad. Med. Sci. and Peking Union Med. Col. 2, A56.
11. Beauchamp, R.D., Townsend, C.M., Jr., Singh, P., Glass, E.J., and Thompson, J.C. (1985) Ann. Surg. 202, 303-309.
12. Singh, P., Le, S., Beauchamp, R.D., Townsend, C.M., Jr., and Thompson, J.C. (1987) Cancer Res. 47, 5000-5004.
13. Matsumoto, M., Park, J., and Yamada, T. (1987) Am. J. Physiol. 252, G143-G147.
14. Baldwin, G.S., Chandler, R., Scanlon, D.B., and Weinstock, J. (1986) J. Biol. Chem. 261, 12252-12257.

15. Singh, P., Chicone, L., Guo, Y-S., Narayan, S., Rajakumar, G., Parekh, D., Townsend, Jr. Gastrointestinal hormone receptors and receptor-regulation. In Gastrointestinal Endocrinology: Receptor and Post-Receptor Mechanisms. (J.C. Thompson, C. Cooper, G.H. Greeley, Jr., P.L. Rayford, P. Singh, C.M. Townsend, Jr., Eds). Academic Press, New York (In Press).
16. Singh, P., Chicone, L. (1988) The FASEB Journal 2, 2551.
17. Chicone, L., Townsend, Jr., C.M., Thompson J.C., Singh, P. (1988) Gastroenterology 94, A67.
18. Singh, P., Rae-Venter, B., Townsend, C.M., Khalil, T., and Thompson, J.C. (1985) Am. J. Physiol. 249, G761-G769.
19. Scatchard G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
20. Laemmli, U.K. (1970) Nature 227, 680-685.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Soll, A.H., Yamada, T., Park, J., Amirian, D., and Thomas, L. (1984) Dig. Dis. Sci. 29, A80.
23. Chang, R.S.L., Lotti, V.J., Keegan, M.E., and Kunkel, K.A. (1986) Biochem. Biophys. Res. Comm. 134, 895-899.
24. Ramani, N., and Praissman, M. (1989) Endocrinology 123, 1881-1887.
25. Kris, R.M., Hazan, R., Villines, J., Moody, T.W., and Schlessinger, J. (1987) J. Biol. Chem. 262, 11215-11220.
26. Miller, L.J. Biochemical characterization of receptors for cholecystokinin family hormones. In Gastrointestinal Endocrinology: Receptor and Post-Receptor Mechanisms. (J.C. Thompson, C. Cooper, G.H. Greeley, Jr., P.L. Rayford, P. Singh, C.M. Townsend, Jr., Eds). Academic Press, New York (In Press).
27. Pearson, R.K., Hadac, E.M., and Miller, L.J. (1989) Am. J. Physiol. 256, G1005-G1010.