Characterization of Functional Receptors for Gastrointestinal Hormones on Human Colon Cancer Cells


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ABSTRACT

Studies demonstrate that some colon cancers possess receptors for various gastrointestinal hormones or neurotransmitters, the occupation of which can affect growth. These results are limited because frequently only a small number of tumors are studied, only 1 or 2 receptors are sought, and the effect on cell function is not investigated. In the present study, 10 recently characterized human colon cancer cell lines were studied to determine whether they possess receptors for any of 12 different gastrointestinal hormones or neurotransmitters and to determine whether these receptors mediate changes in cellular function. Each of the cell lines exhibited receptors for at least one radioligand. Receptors for vasoactive intestinal peptide (VIP) and muscarinic cholinergic agents occurred on 60%, bombesin and gastrin on 30%, β-adrenergic agents and gastrin-releasing peptide (GRP) on 20%, and somatostatin, opiates, neuromedin B, and substance P on 10%. Analysis of [3H]methylscopolamine binding revealed a Kd of 0.2 μM for N-methylscopolamine with a binding capacity of 2500 sites/cell. With the agonist carbachol, the receptor exhibited 2 classes of binding sites: one of high affinity (Kd 55 μM) representing 75% of the binding sites and one of low affinity (Kd 0.3 μM) representing 25% of the binding sites. Analysis of [125I]-Tyr3 bombesin binding revealed a receptor of high affinity (Kd 2.1 μM) with a binding capacity of 3300 sites/cell. Inhibition of binding by agonists revealed relative potencies of [125I]-Tyr3 bombesin > GRP > neuromedin B, and two recently described antagonists were similar in potency to GRP. Analysis of [125I]-VIP binding revealed a receptor having 2 classes of binding sites: one of high affinity (Kd 3.6 nM) and one of low affinity (Kd 1.7 μM) which represented the majority of the 5.5 × 106 binding sites/cell. The relative potencies of agonists were VIP > helodermin > peptide histidine methionine > secretin. Evaluation of biological activity mediated by the muscarinic cholinergic and bombesin receptors revealed an increase of intracellular calcium and of inositol triphosphate by specific receptor agonists. The presence or absence of receptors detected by binding correlated closely with the ability of selective receptor agonists to alter cell function. These results demonstrate the presence of several different receptors for gastrointestinal hormones or neurotransmitters, some described for the first time, on human colon cancer cell lines, including bombesin-related peptides, VIP, somatostatin, substance P, β-adrenergic agents, calcitonin gene-related peptide, gastrin, muscarinic cholinergic agents, and opiates. These receptors are functional because occupation by selective agonists altered intracellular mediators. These results suggest that it will be important to extend these studies to evaluate growth effects.

INTRODUCTION

Colon cancer is one of the most common solid tumors in the United States, second in frequency only to lung cancer (1). Although great strides have been made in the early detection and prevention of colon cancer using occult blood testing and colonoscopic polypectomy (2), the therapeutic options for advanced disease are limited in their efficacy. At present, the 5-year survival of patients with advanced colon cancer remains low (3). This has prompted investigators to seek new modalities of treatment effective in controlling the growth and degree of differentiation of these tumors.

Recently, receptors for a number of gastrointestinal hormones have been found on several tumors such as breast and prostate and small cell lung carcinoma (4–6). The occupation of some of these receptors by agonists or antagonists has been shown to modulate tumor cell growth and function (7). Several reports have demonstrated the presence of receptors for various gastrointestinal hormones on human colon cancer cell lines (8–11), and a few have demonstrated the biological activity of these receptors by studying increases in cAMP3 or phospholipid turnover (10–12). In addition, a number of reports have demonstrated that some of these hormones can affect the growth of colon cancer cells (13–16). For example, gastrin has a trophic effect on several human colon cancer cell lines, and some authors have suggested that gastrin may be an autocrine growth factor for colon cancer cell lines (13–17). Somatostatin has been reported to inhibit the trophic effects of gastrin (15). These reports are limited because only a small number of cell lines were examined, the presence of only a few specific receptors was investigated, the effect of receptor-ligand interaction on cell function was not always examined, and there was often no attempt to correlate the presence or absence of receptors with an effect on cell function. This has made it difficult to determine whether the presence of gastrointestinal receptors on colon cancer cell lines is common or not, whether colon cancer cells possess a number of different classes of receptors for gastrointestinal hormones, or whether there is one particular receptor that is present on all colon tumors.

To address these uncertainties, we studied 10 newly characterized human colon cancer cell lines for the presence of hormone receptors using 12 different radiolabeled ligands. These receptors are characterized pharmacologically on these cell lines, their binding characteristics compared to similar receptors on normal physiological tissues, and the effect of receptor occupation on cell function examined by studying intracellular mediators. These data provide information for future studies examining these hormones in modulating colon tumor cell growth or differentiation.

MATERIALS AND METHODS

[125I]-BH-CCK-8 (2200 Ci/mmol), [125I]-BH-SP (2200 Ci/mmol), [125I]-gastrin 17–1 (2200 Ci/mmol), [125I]-VIP (2200 Ci/mmol), [125I]-OH-BZP (2200 Ci/mmol), and [7H]-methylscopolamine (70–87 Ci/mmol) were

Received 7/30/91; accepted 12/13/91.

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3 The abbreviations used are: cAMP, cyclic AMP; CCK-8, cholecystokinin octapeptide; [125I]-BH-CCK-8, [125I]-Bolton Hunter-labeled CCK-8; [125I]-BH-SP, [125I]-Bolton Hunter-labeled substance P; VIP, vasoactive intestinal peptide; [125I]-OH-BZP, [125I]-hydroxybenzylpindolol; NMB, neuromedin B; [125I]-BH-NMB, [125I]-Bolton Hunter-labeled NMB; GRP, gastrin-releasing peptide; CGRP, calcitonin gene-related peptide; NMS, methylscopolamine bromide; IC50, 50% inhibitory concentration; [Ca2+]i, concentration of intracellular cytosolic calcium; 1P3, inositol trisphosphate.
obtained from New England Nuclear (Boston, MA). ^25I-[Tyr^4]bombesin (2200 Ci/mmol), ^125I-BH-NMB (2200 Ci/mmol), ^125I-secretin (2200 Ci/mmol), and ^125I-L-CGRP (2200 Ci/mmol) were prepared using the methods described previously (18-21). ^125I-Tyr^11-somatostatin-14 (2000 Ci/mmol) and (15,16-^3H)Heporphine (30-60 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). CCK-8 was obtained from Research Plus (Bayonne, NJ); [Tyr^4]bombesin and GRP were obtained from Bachem, Inc. (Torrance, CA); gastrin-I, NMB, VIP, secretin, substance P, somatostatin, helodermin, peptide histidine methionine-27, and CGRP were obtained from Peninsula Laboratories (Belmont, CA); pindolol, carbamylcholine chloride (carbachol), and NMS were obtained from Sigma Chemical Co. (St. Louis, MO). The bombesin analogues were synthesized and characterized as described previously (22-24). Etoporphine was from the Laboratory of Chemistry, NIH, Bethesda, MD. Media and sera were obtained from Grand Island Biological Co. (Grand Island, NY).

Cell Culture. Ten human colon cancer cell lines, recently established as previously described, were examined (25). Relevant clinical information concerning the primary site of tumors, culture site of tumors, and degree of tumor differentiation is provided in Table 1. Cell lines were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum. Adherent cultures were passaged weekly at subconfluence after trypsinization. Nonadherent cultures were passaged weekly by transfer of floating multicellular aggregates. Cultures were maintained in incubators at 37°C in an atmosphere of 5% CO₂ and 95% air.

Binding of Radiolabeled Ligand. Adherent cells were scraped from the flask with a rubber policeman at least 72 h after last passage using trypsin. Cells were washed twice in five-fold volume % of media, spun down at 1000 x g for 5 min, and resuspended in standard incubation buffer consisting of 50 mM Tris buffer, 0.1% bacitracin, 5 mM MgCl₂, 250 mM NaCl, 7.7 mM KCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 4 μg/ml leupeptin, 2 μg/ml chymostatin, and 0.1% bovine serum albumin at pH 7.4. After washing, the viability of the cells was assessed by trypan blue exclusion and was >90%, with no evidence of cell lysis. Cells at a concentration of 15 x 10⁶ cells/ml (range, 5-25 x 10⁶ cells/ml), in a volume of 0.5 ml were incubated with radioligand at a concentration of 50 pm for ^125I-BH-CCK-8, ^125I-[Tyr^4]bombesin, ^125I-BH-NMB, ^125I-VIP, ^125I-secretin, and ^125I-OH-BZP, 100 pm for ^125I-gastrin 1-71 and ^125I-[Tyr^11]somatostatin-14; 125 pm for ^125I-BH-SP and ^125I-CGRP; 0.6 nm for [^3H]NMS; and 1.5 nm for [15,16-^3H]Etoporphine, for the times and temperatures indicated. After incubation with iodinated radioligand, 0.133 ml of cells were sampled, spun down through standard incubation buffer containing 4% bovine serum albumin in a Beckman microfuge at 10,000 x g for 1 min, and then washed twice. The pellet was then counted in an Auto-Gamma 5000 series gamma counter (Packard Instrument Corp., Sterling, VA). After incubation with a tritiated radioligand, cells were sampled, filtered using glass microfiber filters (GF/C; Whatman International Ltd., Maidstone, England), and then counted in a liquid scintillation spectrometer (Packard Instrument Corp., Sterling, VA).

Non-saturable binding was the amount of radioactivity associated with the cells when the incubation contained radioligand plus the unlabeled peptide in concentrations of 1–10 μM. Values shown for saturating binding are those measuring binding with radioligand alone (total binding) minus the nonsaturable binding. All values are for saturable binding unless stated otherwise. Dissociation constants were determined by the nonlinear, least-squares curve-fitting program (LIGAND) (26). The inhibitory constant values for antagonist binding and their relationship to the IC₅₀ were calculated by the method of Cheng and Prusoff (27). The number of binding sites was determined by the curve-fitting program (LIGAND) (26) and expressed as binding sites/cell.

To establish that the ligands would bind to the appropriate receptor, the activity of all radioligands was tested for binding in cell systems known to exhibit receptors for the ligand. Dispersed cells from guinea pig pancreatic acini were used for the following ligands: with ^125I-[Tyr^4]bombesin, 1% of the total counts added bound with a nonsaturable binding of <0.5% of the total counts bound; with ^125I-secretin 20% of the total counts bound with a nonsaturable binding of <15% of the total counts bound; with ^125I-gastrin 4% of the total counts added bound with a nonsaturable binding of <30% of the total counts bound; with ^125I-CCK-8, 10% of the total counts added with a nonsaturable binding of <15% of the total counts bound; and with ^125I-[Tyr^11]somatostatin, 10% of the total counts added bound with a nonsaturable binding of <20% of the total counts bound. Guinea pig gastric smooth muscle cells were used for the following ligands: with ^125I-OH-BZP, 3% of the total counts added bound with a nonsaturable binding of <15% of the total counts bound; with ^125I-CGRP, 4% of the total counts added bound with a nonsaturable binding of <15%; and with [^3H]Etoporphine, 1.2% of the total counts added bound with a nonsaturable binding of <25% of the total counts bound. Chief cells from guinea pig stomach were used for [^3H]NMS, which bound 5% of the total counts added with a nonspecific binding of <10% of the total counts bound. Tissue sections from rat esophagus were used for ^125I-BH-NMB, which bound 2000 cpm/sec (50 pm radioligand added) with a nonsaturable binding of <15% of the total counts bound.

The human colon cancer cell lines studied exhibited a nonsaturable binding of <15% in all cases in which binding was significant for all radioligands except pindolol, for which the nonsaturable binding was <30% of the total counts bound.

Intracellular Calcium Mobilization. Intracellular calcium concentrations were measured using the fluorescence indicator fura-2/AM. Cells were loaded with fura-2/AM (Molecular Probes, Inc., Eugene, OR) at 2 μM in a volume containing 3.5 x 10⁶ cells/ml. The cells were incubated in the dark for 40 min at 37°C, washed in standard incubation buffer at pH 7.4 three times, and resuspended in standard incubation buffer in the original volume. The fluorescence of fura-2-containing cells was measured with a dual wavelength excitation fluorimeter (Photon Technology International, Inc., So. Brunswick, NJ). The wavelengths were 340 and 380 nm for excitation and 500 nm for emission. [Ca²⁺]ᵢ was calculated as previously described by Grynkiewicz et al. (28), using the formula:

\[ [Ca^{2+}]_i = K_d \times (R - R_{min}) \times \frac{R_{max} - R}{S_F} \]

where Kₚ is the affinity of fura-2 for Ca²⁺, determined to be 225 nm, R is F₅₀/F₃₀ is the ratio of the fluorescence with the two excitation wavelengths, Rₕ is the ratio of F₃₁₀/F₃₂₀ in a saturated calcium environment after addition of 0.1% Triton, Rₚ is the fluorescence ratio at virtually zero calcium by the addition of 25 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, S_F is the F₃₂₀ at zero [Ca²⁺]ᵢ, and Sₚ is the F₃₂₀ at saturated [Ca²⁺]ᵢ. All experiments were performed with constant mixing by a magnetic stirrer under the cuvette holder.

IP₃ Determination. Changes in IP₃ (1, 4, 5) were measured using the IP₃ (1, 4, 5) radioreceptor assay system from Amersham, as described previously (29). Cells were incubated at a concentration of 20 x 10⁶ cells/ml in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at pH 7.4. Incubations were at 37°C and were terminated by adding...
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trichloroacetic acid. After centrifugation for 15 min at 1000 x g, 0.5 ml of the samples was extracted five times with 2.5 ml of water-saturated diethyl ether and neutralized with KHCO₃, and 100 µl of the samples was analyzed in the radioreceptor assay.

DNA Determination. Cellular DNA was determined using the diphenylamine method described by Burton (30). Duplicate samples of cell suspension were assayed for DNA.

Statistical Analysis. Differences were analyzed using Student’s t test. Differences of P < 0.05 were considered significant.

RESULTS

Radiolabeled Ligand-binding Studies. Six of the 10 cell lines exhibited a significant amount of binding of ³²P-[Tyr⁴]bombesin with a mean value of 67 fmol/µg DNA (range, 19-111 fmol/µg DNA), and six cell lines exhibited a significant amount of binding of [³H]NMS with a mean value of 1332 fmol/µg DNA (range, 314-3443 fmol/µg DNA) (Table 2). Three cell lines exhibited a significant amount of binding of [¹²⁵I]-[Tyr⁴]bombesin with a mean value of 126 fmol/µg DNA (range, 17-294 fmol/µg DNA), and three cell lines exhibited a significant amount of binding of [¹²⁵I]-BH-CKK-8 with a mean value of 52 fmol/µg DNA (range, 16-70 fmol/µg DNA). Two cell lines exhibited a significant amount of binding of [¹²⁵I]-OH-BZP with values of 55 and 294 fmol/µg DNA, and two cell lines exhibited a significant amount of binding of [¹²⁵I]-CGRP with values of 4 and 44 fmol/µg DNA. One cell line exhibited a significant amount of binding to [¹²⁵I]-BH-NMB with a value of 3 ± 0.002 fmol/µg DNA (mean ± SEM), one cell line exhibited a significant amount of binding to [¹²⁵I]-[Tyr⁴]somatostatin with a value of 246 ± 1.5 fmol/µg DNA, one cell line exhibited significant binding to [¹²⁵I]-BH-SP with a value of 22 ± 0.1 fmol/µg DNA, one cell line exhibited significant binding to [¹²⁵I]-gastrin 17-1 with a value of 43 ± 0.2 fmol/µg DNA, and one cell line exhibited significant binding to [³H]heterorphine with a value of 6784 ± 115 fmol/µg DNA. None of the 10 cell lines exhibited significant binding to [³H]-VIP.

All of the cell lines exhibited binding for at least one of the radioligands studied (Table 2). Cell lines NCI-H630 and SNU-C4 exhibited binding for only one radioligand each, cell lines NCI-H508, NCI-H768, and SNU-C1 exhibited binding for 2 radioligands each, cell lines NCI-H498, NCI-H548, NCI-H747, and SNU-C5 exhibited binding for 3 radioligands each, and NCI-H716 exhibited binding for 7 of the radioligands studied.

To further examine the interaction of [³H]NMS with receptors on these cell lines, we studied the binding characteristics of this radioligand on cell line H-508. At 22°C, binding was maximal by 45 min, remained constant for the next 45 min, and then decreased thereafter (Fig. 1). Increasing the incubation temperature from 22 to 37°C caused a more rapid time course with maximal binding by 10 min and a progressive decrease thereafter, such that, after 120 min of incubation, binding was 50% that at 22°C. At 4°C, binding was maximal at 45 min, at which time it was 50% that of binding at 22°C, and was constant for an additional 75 min. Adding 10 µM NMS reduced binding of [³H]NMS by 88, 93, and 93% of maximal binding at 4, 22, and 37°C, respectively.

The muscarinic cholinergic agonist, carbamylcholine, and the muscarinic cholinergic antagonist, NMS, were tested for their abilities to inhibit binding of [³H]NMS. With NMS, detectable inhibition occurred at a concentration of 100 µM, half-maximal inhibition at 1 µM, and complete inhibition of [³H]NMS binding at 1 µM (Fig. 2). With carbamylcholine, detectable inhibition occurred at 10 µM, half-maximal inhibition at 300 µM, and

Table 2  Radiolabeled peptides saturably bound to human colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No.</th>
<th>Radiolabeled peptide bound (fmol/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H498</td>
<td>3</td>
<td>[¹²⁵I][Tyr⁴]bombesin (67 ± 1), [¹²⁵I]-BH-CKK-8 (16 ± 1), [³H]NMS (399 ± 3)</td>
</tr>
<tr>
<td>NCI-H508</td>
<td>2</td>
<td>[¹²⁵I]-VIP (111 ± 2), [³H]NMS (1561 ± 22)</td>
</tr>
<tr>
<td>NCI-H548</td>
<td>3</td>
<td>[¹²⁵I]-gastrin 17-1 (43 ± 1), [¹²⁵I]-BH-CKK-8 (70 ± 2), [³H]NMS (314 ± 1)</td>
</tr>
<tr>
<td>NCI-H630</td>
<td>1</td>
<td>[¹²⁵I]-VIP (76 ± 3)</td>
</tr>
<tr>
<td>NCI-H716</td>
<td>7</td>
<td>[¹²⁵I][Tyr⁴]bombesin (294 ± 7), [¹²⁵I]-BH-NMB (35 ± 0.1), [¹²⁵I][Tyr⁴]somatostatin (246 ± 2), [¹²⁵I]-BH-SP (22 ± 1), [³H]CGRP (44 ± 1), [³H]-BH-CKK-8 (69 ± 1), [³H]NMS (3443 ± 58)</td>
</tr>
<tr>
<td>NCI-H747</td>
<td>3</td>
<td>[¹²⁵I]-VIP (19 ± 1), [³H]-OH-BZP (55 ± 1), [³H]heterorphine (6784 ± 115)</td>
</tr>
<tr>
<td>NCI-H768</td>
<td>2</td>
<td>[¹²⁵I]-OH-BZP (294 ± 16), [³H]NMS (1002 ± 24)</td>
</tr>
<tr>
<td>SNU-C1</td>
<td>2</td>
<td>[¹²⁵I]-VIP (38 ± 1), [³H]NMS (1270 ± 41)</td>
</tr>
<tr>
<td>SNU-C4</td>
<td>1</td>
<td>[¹²⁵I]-VIP (99 ± 1)</td>
</tr>
<tr>
<td>SNU-C5</td>
<td>3</td>
<td>[¹²⁵I][Tyr⁴]bombesin (17 ± 1), [¹²⁵I]-VIP (61 ± 2), [³H]-CGRP (4.4 ± 0.1)</td>
</tr>
</tbody>
</table>

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Fig. 1. Time course of binding of [³H]NMS to colon cell line NCI-H508. Cells were incubated with 0.6 nM [³H]NMS for the times indicated at 4, 22, or 37°C alone or with 10 µM NMS. Results are the percentages of the added radioligand bound at the times indicated. Points, means from 4 separate experiments; vertical bars, SEM.

Fig. 2. Ability of NMS and carbamylcholine to inhibit binding of [³H]NMS to colon cell line NCI-H508. Cells were incubated at 22°C for 45 min with 0.6 nM [³H]NMS plus the indicated concentrations of ligands. Binding is expressed as percentage of [³H]NMS that was saturably bound in the absence of ligand. Points, means from at least 4 experiments; vertical bars, SEM.

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Table 3 Results of computer analysis of the ability of the muscarinic cholinergic antagonist, N-methylscopolamine, or agonist, carbachol, to inhibit binding of \textsuperscript{3}H-NMS to the human colon cancer cell line NCI-H716

<table>
<thead>
<tr>
<th>Agent</th>
<th>(K_a) high</th>
<th>(K_a) low</th>
<th>No. of binding sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>0.2 ± 0.04 nM</td>
<td>0.29 ± 0.04 nM</td>
<td>2499 ± 153 (100)</td>
</tr>
<tr>
<td>Carbamylcholine</td>
<td>55.3 ± 6.5 (\mu)M</td>
<td>33.3 ± 0.03 nM</td>
<td>1874 ± 178 (75)</td>
</tr>
</tbody>
</table>

Values are means ± SEM of at least 4 experiments. \(K_a\) values and number of binding sites were obtained by analysis of the dose-inhibition curves of \textsuperscript{3}H-NMS binding shown in Fig. 2 using the nonlinear, least-squares curve fitting program, LIGAND (26). The number of binding sites was converted to sites/cell after determining the number of cells/ml of incubation volume. Numbers in parentheses, \% of total.

(\(K_a\) 0.33 ± 0.03 nm) (Table 3). The computer analysis of the dose-inhibition curve of \textsuperscript{3}H-NMS binding by NMS was best fit by a model having a single class of binding sites, demonstrating that the antagonist could not distinguish between the muscarinic cholinergic receptors having high and low affinities for the agonists. The analysis demonstrated 2499 ± 153 binding sites/cell (\(n = 6\)) having a \(K_a\) of 0.20 ± 0.04 nM (Table 3).

To examine the interaction of \textsuperscript{125}I-[Tyr\textsuperscript{4}]bombesin on these human colon cancer cells, we studied the binding characteristics of this radioligand on cell line NCI-H716 (Fig. 3). [Tyr\textsuperscript{4}] bombesin, an analogue, has previously been shown (18) to be a high-affinity radioligand useful for identifying receptors that interact with bombesin and structurally related peptides. At 22°C, there was a steady increase in binding such that maximal binding occurred at 90 min. Increasing the incubation temperature to 37°C caused a more rapid time course with a leveling off of binding at 45 min for the next 75 min, such that the amount of binding at 120 min was 85% that at 22°C. Decreasing the incubation temperature to 4°C caused a decrease in binding such that maximal binding was not reached until 120 min, at which time it was 30% that at 22°C. Adding 1 \(\mu\)M [Tyr\textsuperscript{4}] bombesin to the incubation reduced binding of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin by >95% at all temperatures (Fig. 3).

To characterize the subtype of bombesin receptor present, various bombesin receptor agonists and antagonists were tested for their abilities to inhibit binding of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin. Of the agonists tested, [Tyr\textsuperscript{4}] bombesin was the most potent (Fig. 4). Detectable inhibition of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin binding occurred with 0.1 nM [Tyr\textsuperscript{4}] bombesin, half-maximal inhibition with 3 nM, and complete inhibition with 100 nM (Fig. 4). Computer analysis of the dose-inhibition curve of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin binding by [Tyr\textsuperscript{4}] bombesin was best fit by a model having a single class of binding sites. The analysis demonstrated 3312 ± 301 binding sites/cell (\(n = 3\)) of high affinity (\(K_a 2.1 \text{ nM}\)). For inhibiting binding of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin, [Tyr\textsuperscript{4}] bombesin was 3-fold more potent than GRP (\(K_a 6.6 ± 1.3 \text{ nM}\), \(n = 3\); Table 4) and 300-fold more potent than NMB (\(K_a 700 ± 200 \text{ nM}\), \(n = 3\); Table 4). Of the two recently described (23) bombesin receptor antagonists tested, [D-Phe\textsuperscript{6}]-Bn(6-13)propylamide was the more potent, having a \(K_a\) of 5.9 ± 0.8 nM, which is only 3-fold less potent than [Tyr\textsuperscript{4}] bombesin in inhibiting the binding of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin. [D-Phe\textsuperscript{6}]-Leu\textsuperscript{12}, \(\psi(CH_3)NH\)Cpa\textsuperscript{14}]Bn(6-14) was also potent, having a \(K_a\) of 7.0 ± 0.8 nM (\(n = 3\); Fig. 5, Table 4), which is 3.3-fold less potent than [Tyr\textsuperscript{4}] bombesin (Table 4).

To examine the interaction of \textsuperscript{125}I-VIP on these human colon cancer cell lines, we studied the binding characteristics of this radioligand on cell line SNU-C4. At 37°C, there was a steady increase in binding, reaching a plateau at 45 min, remaining stable for an additional 45 min, and then decreasing (Fig. 5). Decreasing the incubation temperature to 22°C slowed the time course such that a plateau was reached at 60 min, at which point binding was 96% that at 37°C. Decreasing the incubation temperature to 4°C caused a decrease in the rate of binding.

Table 4 Ability of various bombesin-related peptides to inhibit binding of \textsuperscript{125}I-[Tyr\textsuperscript{4}] bombesin to human colon cancer cell line NCI-H716

<table>
<thead>
<tr>
<th>Peptide</th>
<th>(K_a) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Tyr\textsuperscript{4}] bombesin</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>GRP</td>
<td>6.6 ± 1.3</td>
</tr>
<tr>
<td>Neuromedin B</td>
<td>700.0 ± 200.0</td>
</tr>
<tr>
<td>[D-Phe\textsuperscript{6}]-Bn(6-13)propylamide</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>[D-Phe\textsuperscript{6}]-Leu\textsuperscript{12}, (\psi(CH_3)NH)Cpa\textsuperscript{14}]Bn(6-14)</td>
<td>7.0 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SEM of at least 4 experiments. \(K_a\) values were calculated according to the method of Cheng and Prusoff from the dose-inhibition curves in Fig. 4.
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Fig. 5. Time course of binding of 125I-VIP to colon cell line SNU-C4. Cells were incubated with 50 pM 125I-VIP for the times indicated at 4, 22, or 37°C alone or with 1 nM VIP. Results are percentages of the added radioligand bound at the times indicated. Points, means from 5 separate experiments; vertical bars, SEM.

Fig. 6. Abilities of various VIP receptor agonists to inhibit binding of 125I-VIP to colon cell line SNU-C4. Cells were incubated at 22°C for 45 min with 50 pM 125I-VIP plus the indicated concentrations of agonists. Binding is expressed as percentage of 125I-VIP that was saturably bound in the absence of unlabeled agonist. Points, means from at least 5 experiments; vertical bars, SEM.

Table 5 Ability of VIP-secretin-related peptides to interact with VIP receptors on colon cancer cell line SNU-C4

<table>
<thead>
<tr>
<th>Agent</th>
<th>Binding of 125I-VIP IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td>Helodermin</td>
<td>20.2 ± 0.8</td>
</tr>
<tr>
<td>Peptide histidine methionine</td>
<td>139 ± 7</td>
</tr>
<tr>
<td>Secretin</td>
<td>2121 ± 82</td>
</tr>
</tbody>
</table>

Values are means ± SEM of at least 5 experiments. IC50 is the concentration of agonist required to inhibit saturable binding of 125I-VIP by 50%. Values were calculated from the experiments shown in Fig. 6 by the method of Cheng and Prusoff (27).

such that the amount of binding at 120 min was only 17% that at 37°C. Adding 1 μM VIP to the incubation reduced binding of 125I-VIP by 97, 96, and 80% at 37, 22, and 4°C, respectively (Fig. 5).88

To characterize the 125I-VIP binding sites further, various VIP-related peptides were tested for their abilities to inhibit binding of 125I-VIP (Fig. 6). VIP was the most potent with detectable inhibition at 1 nM, half-maximal inhibition at 6 nM, and complete inhibition at 30 μM. Computer analysis of this broadly based dose-inhibition curve of 125I-VIP binding by VIP was best fit with a model having 2 classes of binding sites. The analysis demonstrated 5.5 × 105 binding sites/cell (n = 6) of which 20,000 ± 4,200 binding sites/cell (n = 6) had a high affinity for VIP (Kd 3.6 nM) and the remainder had a low affinity for VIP (Kd 1.7 μM). Helodermin caused a detectable inhibition of 125I-VIP binding at 3 nM (Fig. 6, Table 5) and was 4-fold less potent than VIP (IC50, 20.2 nM, Table 5), peptide histidine methionine was 25-fold less potent than VIP with an IC50 of 139 nM and secretin as 400-fold less potent than VIP with an IC50 of 2121 nM (Table 5).

Studies of Changes in Cytosolic Calcium Concentration. Immunoreactive bombesin-like peptides, as well as other gastrointestinal peptides and neurotransmitters, have been localized to intestinal epithelial cells as well as other gastrointestinal tissues (31–40). Binding of a number of these agents such as bombesin, CCK-8, or muscarinic cholinergic agents to their specific receptors has been shown to alter intracellular calcium (7, 33, 35). To examine whether occupation of these various receptors on human colon carcinoma cell lines resulted in similar biological activity, we studied the ability of [Tyr4]bombesin or carbamylcholine to alter intracellular calcium in the human cancer cell line, NCI-H716. [Tyr4]bombesin caused a rapid transient increase in [Ca2+]i (Fig. 7). The increase was detectable at 0.1 nM with a 12 ± 3% (n = 6) increase of [Ca2+]i above baseline (Fig. 8). Maximal [Ca2+]i mobilization was detected with 1 μM [Tyr4] bombesin, giving a 266 ± 15% (n = 6) increase above baseline, and half-maximal stimulation was detected at 20 nM (Fig. 8).

Receptors for muscarinic cholinergic agents were also found on...
effects on intracellular calcium by interacting with bombesin baseline (Fig. 7). To establish that [Tyr4]bombesin was in fact mediating its rapid transient increase in [Ca2+]ᵢ of 279 ± 22% (n = 6) above baseline (Fig. 7). When 0.1 μM [Tyr4]bombesin was added to NCI-H716 cells, the [Ca2+]ᵢ increased by 259 ± 21% (n = 6) above baseline (Figs. 7 and 8). The addition of the specific bombesin receptor antagonist, [D-Phe⁶]bombesin(6-13)methyl ester (24) to inhibit the action of bombesin (Fig. 7). When 0.1 μM [Tyr4]bombesin was added to NCI-H716 cells, the [Ca2+]ᵢ increased by 259 ± 21% (n = 6) above baseline (Figs. 7 and 8). The addition of the specific bombesin receptor antagonist, [D-Phe⁶]Bn(6-13)methyl ester, caused no increase in [Ca2+]ᵢ but totally blocked the increase in cytosolic calcium caused by 0.1 μM [Tyr4]bombesin added immediately after the antagonist (Fig. 7). The inhibitory action of the antagonist was specific for the bombesin receptor because the antagonist did not effect the increase in cytosolic calcium caused by carbachol (Fig. 7).

To determine whether a cell line without detectible bombesin or muscarinic cholinergic receptors responded to these agents, we examined cell line SNU-C4 which does not exhibit receptors for either bombesin or muscarinic cholinergic agents. There was no increase in [Ca2+]ᵢ detected in this cell line with 1 μM [Tyr⁴]bombesin or 1 mM carbachol (data not shown).

**Cellular Inositol Triphosphate Studies.** Bombesin and muscarinic cholinergic agents have been reported to activate phospholipase C, cause the breakdown of phospholipids, and increase phosphoinositides in intestinal epithelial and other cells (7, 10, 11). To determine whether receptor occupation by these agents had a similar effect on human colon cancer cell lines, we studied the ability of the bombesin receptor agonist, [Tyr⁴] bombesin, or the muscarinic cholinergic receptor agonist, carbachol, to increase IP3(1, 4, 5), the biologically active IP3 isomer which alters cellular calcium. Also, to assess whether the presence of receptors for these agents correlated with the ability to alter IP3(1, 4, 5), 5 s, colon cell lines with and without detected receptors for these agents were tested. [Tyr⁴]bombesin (1 μM) in NCI-H716 cells, which possess bombesin receptors, caused a 4-fold increase above baseline in IP3(1, 4, 5) within 5 s (Table 6). Carbachol (1 mM) in NCI-H716 cells, which also have receptors for muscarinic cholinergic agents, also caused a 4-fold increase in IP3(1, 4, 5) within 5 s (Table 6). The human colon cell line, NCI-H508, which has receptors for muscarinic cholinergic agents but not for bombesin, responded to 1 mM carbachol with a 2-fold increase in IP3(1, 4, 5) within 5 s but did not respond to 1 μM [Tyr⁴]bombesin (Table 6). SNU-C4 cells, which do not have receptors for muscarinic cholinergic agents or for bombesin, did not respond to these agents with an increase in IP3(1, 4, 5) (Table 6).

**DISCUSSION**

The present results demonstrate that human colon cancer cell lines frequently possess functional receptors for a number of gastrointestinal hormones or neurotransmitters. Prior studies (8-11) of the presence of these receptors on human colon cell lines or colon cancers were limited in a number of ways. Frequently, only one receptor on an individual human colon cancer cell line or cancer tissue was studied, making it difficult to determine whether the presence of these receptors was a common or an uncommon finding. The effect of receptor occupancy by agonists on cell function was not always examined, and there was often no attempt to correlate the presence or absence of receptors with an effect on cell function. With the recent availability of a number of well-characterized human colon cancer cell lines, it has become possible to systematically address each of these points. Ten well-characterized human colon cell lines were examined for the presence of functional receptors for gastrointestinal peptides or neurotransmitters.

Each of the 10 cell lines tested exhibited binding sites for at least one ligand, and the only ligand that did not exhibit a binding site on any of the cell lines was radiolabeled secretin. The binding of the 12 radiolabeled ligands tested on the 10 colon cancer cell lines could, theoretically, reveal a maximum of 120 receptors if all of the cell lines bound all of the ligands. A total of 27 receptors of a possible 120 (23%) were exhibited, demonstrating that the presence of receptors for gastrointestinal hormones and neurotransmitters on human colon cancer cell lines is not an uncommon occurrence.

The binding characteristics of [3H]NMS support the presence of a specific receptor for muscarinic cholinergic agents which is similar to muscarinic cholinergic receptors previously described on intestinal epithelium (35, 36, 40). Although there are at least 5 different muscarinic cholinergic receptor subtypes, in the present study we did not attempt to distinguish these subtypes. The binding of [3H]NMS was temperature dependent, saturable, and specific. Similar to these other gastrointestinal tissues (33, 34), computer analysis of the binding revealed 2 classes of binding sites which interacted with different affinities with the muscarinic, cholinergic agonist, carbachol, and with the same high affinity with the muscarinic cholinergic antagonist, N-methylscopolamine, or quinuclidinyl benzilate (40). Seventy-five % of these sites had a high affinity for carbachol (Kd 55 μM), and 25% had a low affinity for the agonist (Kd 0.3 mM). These characteristics are very similar to those reported for the muscarinic cholinergic receptor found in a highly enriched preparation of chief cells prepared from guinea pig stomach (34). The muscarinic cholinergic chief cell receptor also exhibits 2 classes of binding sites, 73% with a high affinity (Kd 53 μM) and 27% with a low affinity (Kd 4.6 mM) for carbachol. Further evidence supporting the conclusion that the binding was to a muscarinic cholinergic receptor on human colon cell lines was the ability of the muscarinic cholinergic receptor agonists to elicit biological response. Carbachol only stimulated a change in cytosolic calcium or inositol triphosphates in those cells in which the muscarinic cholinergic receptor was identified by binding studies.

The binding results with [125I-Tyr⁴]bombesin support the conclusion that it is binding to a bombesin receptor in that the binding was time and temperature dependent, saturable, and specific. Interaction was with a single class of high-affinity binding sites (Kd 2.1 nm), exhibiting 3312 binding sites/cell. Although the bombesin receptor has not been previously de-
The bombesin receptor, which is also known as the gastrin-releasing peptide (GRP) receptor, has been extensively studied in various tumor cell systems and is present on human gastrointestinal colon cancer cell lines. This receptor has a high affinity for bombesin (Kd 1.1 nM) with 6444 binding sites/cell and a low affinity for VIP (Kd 3.6 nM) and NMB. The binding characteristics of this receptor are similar to those identified on 3T3 cells, the pancreatic tumor cell line AR42J, and normal pancreatic acinar cells, as well as some areas of the central nervous system. The ability of bombesin to cause an increase of cytosolic calcium and inositol phosphates in human colon cancer cells exhibiting binding of 125I-[Tyr4]bombesin was additional evidence of the presence of a specific bombesin receptor which was functional. That the bombesin binding site identified represented the receptor was suggested by the ability of bombesin to occupy the bombesin receptor and inhibit binding of 125I-[Tyr4]bombesin and to increase intracellular calcium. In addition, the fact that bombesin was altering biological activity through the bombesin receptor was demonstrated, in that the increase of cytosolic calcium was blocked by specific bombesin receptor antagonists which did not elicit a subsequent response to carbachol.

The binding sites identified using 125I-VIP suggest that they represent an interaction with a VIP receptor in that it is saturable, specific, and of the same nature as dispersed pancreatic acini from guinea pig pancreas which exhibits 2 classes of binding sites (9, 47). In both of these cases, as well as in a human intestinal cell line (38), the receptor was of high affinity (Kd 0.12 nM in colon, 2.5 nM in gastric, and 0.13 nM in intestine), but only one binding site was exhibited. This difference raises the possibility that the VIP receptors previously described on human colon cancers (9), which exhibit only a single site, are in fact different from the VIP receptors described here that exhibit 2 sites, but it is more likely that these differences are due to methodological differences. The low-affinity VIP-binding site would not have been demonstrated if a nonsaturable concentration of 0.01–1 μM VIP was used as in the previous studies (9, 38, 47), as opposed to a nonsaturable concentration of 10 μM VIP as was used in these experiments and in the study of pancreatic acini, which demonstrated a 2-site model (46). The biological activity of the VIP receptor on these cell lines has not yet been studied. However, previous reports have described the presence of functional VIP receptors on the human colon cancer cell line, HT-29, that exhibits an increase in cAMP in response to VIP (9, 12).

Because of the limited availability of gastrointestinal tumor cell lines of human origin, only occasional reports of the presence of receptors for gastrointestinal peptides on human tumor cell lines have been published (8–11, 47–50). Human colon cancer cell lines have been reported to exhibit high-affinity receptors for VIP, demonstrated by radioligand binding and a cAMP response on the human colon cell line HT-29 (9, 12). In addition, receptors for muscarinic cholinergic agents were demonstrated by radioligand binding and inositol phosphate turnover on the human colon cell line HT-29 (10, 11), and receptors for gastrin were demonstrated by radioligand binding on the human colon cell line LoVo (8). Human gastric cancer cell lines have been reported to exhibit receptors for muscarinic cholinergic agents demonstrated by radioligand binding on 2 of 4 newly established cell lines (47), receptors for VIP, demonstrated by radioligand binding, and a cAMP response on the cell line HGT-1 (50) and 4 of 4 newly established cell lines (47), receptors for gastrin demonstrated by radioligand binding on several cell lines (51), and receptors for histamine H2-receptor agonists, gastric inhibitory peptide and glucagon by a cAMP response on cell line HGT-1 (52, 53). Human pancreatic cancer cell lines have been reported to exhibit receptors for VIP by radioligand binding and a cAMP response on cell line capan-1 (54) and receptors for muscarinic cholinergic agents by radioligand binding and inositol phosphate formation (55). Of the receptors studied, only those for VIP, gastrin, and muscarinic cholinergic agents have been previously described on human colon cancer cell lines. The present study is the first to demonstrate the presence of receptors for bombesin, CGRP, NMB, somatostatin, substance P, and adrenergic agents, and opiates on human colon cancer cell lines.

During the past several years, much attention has been given to the role of gastrin in promoting the growth of human colon and gastric cancer (8, 13–15). Gastrin has been reported to increase the growth of various human colon cancers or gastric cancers implanted into nude mice and the gastrin/cholecystokinin receptor antagonist, proglumide, to inhibit the growth (15, 16, 56). Gastrin receptor content of colonic neoplasms has been reported to have prognostic significance (57). In other studies, gastrin receptors have been identified by radioligand binding on some human colon and gastric cancer cell lines (8, 51). Although a number of the reports demonstrated the presence of these receptors on only one or 2 cell lines (13, 56), at least 2 reports demonstrated gastrin receptors on 4 or 5 cell lines (14, 51). In our study, gastrin receptors occurred on only 10% of the human cancer cell lines, suggesting that they occur relatively infrequently. These results are in agreement with reports describing gastrin receptors on a small number of tumors (13, 15, 56) but are at variance with reports describing gastrin receptors on a number of human colon cancer cell lines (14, 51). Several explanations could account for this difference. It is possible that receptors were present but not detected because of the methodology, loss of receptors during cell preparation for binding studies such as due to cell lysis and loss of membranes, or the conditions were not optimal for binding. These possibilities seem unlikely because cell integrity was assessed by trypan blue exclusion, cells were rapidly processed.
and the ligands bound well to control cells using similar conditions. In both previous reports, as well as the present study (14, 51), the human colon cancer cell lines were maintained in media containing 5–10% fetal bovine serum; however, different media with different supplementation were used in the different studies, and this possibly may have an effect on the number of receptors present on the cell surface at any given time. We studied 7 cell lines which originated in the United States and 3 which originated in Korea, while Weinstock and Baldwin (51) studied human colon cancer cell lines originating in Japan and Australia, and Watson et al. (14) studied cell lines originating in the United Kingdom as well as other origins. Thus, it is possible that the human colon cancer cell lines derived from tumors in different geographic locations have different biological characteristics. Another possibility could be the length of time that the cell lines have remained in culture. In the report by Watson et al. (14), 4 freshly disaggregated human adenocarcinomas responded to gastrin, 2 newly established colon cancer cell lines responded to gastrin at passage 2 but not passage 6, and 8 established colon cancer cell lines did not respond to gastrin, but 1 of these lines responded to gastrin during synchronization of the cell cycle. The established cell lines in the report by Watson et al. (14) were passaged >500 times, a substantially high number of passages. This latter study suggests that the ability of some colon tumor cell lines to respond to a peptide and perhaps the presence of its receptor is a dynamic process that may change during the course of the cell line. This raises the possibility that the percentage of human colon cancer cell lines demonstrating gastrin receptors in this study may actually be an underestimate of the presence of gastrin receptors in the original human colon cancer tissue from which the tumor cell line was derived. A recent study of surgical specimens from 67 patients with primary colon cancer supports this possibility (57). In this study (57), 57% of the specimens examined were reported to possess gastrin receptors. Of further interest is the number and variety of receptors for gastrointestinal hormones or neurotransmitters demonstrated on the human colon cancer cell lines, compared to those previously reported on the human gastric cancer cell lines, using the same ligands as in the present study (47). A greater number of receptors for hormones and neurotransmitters were exhibited on human colon cancer cell lines, i.e., 11 of the 12 (92%) different receptors sought were present on at least one colon cell line, compared to only 2 of 9 (22%) receptors found on human gastric cancer cell lines. At present, the basis for this difference is unclear, as is the possibility that gastrointestinal hormones may have a greater effect on colon cancer cell growth.

As mentioned earlier, one of the prime interests in establishing the presence of receptors for gastrointestinal peptides or neurotransmitters on gastrointestinal tumor cell lines is to determine whether they exhibit biological activity and to establish whether alteration of intracellular mediators has a mitogenic effect on cell differentiation. This has been shown to be the case for the gastrointestinal peptide, bombesin, which interacts with high-affinity receptors on small cell lung carcinoma (7, 58). Many of the classic small cell lung carcinoma cell lines exhibit receptors for bombesin, and bombesin has been shown to modulate an autocrine growth effect which can be inhibited by bombesin receptor antagonists or by bombesin antibodies (6, 7, 59, 60). The present study demonstrates that bombesin receptors and muscarinic cholinergic receptors on the human colon cancer cell lines could alter intracellular mediators in the tumor cells. This suggests that occupation of the GRP-prefer-
27. Cheng, Y. C., and Prusoff, W. H. Relationship between the inhibition constant and the concentration of inhibitor which causes 50 per cent
activity.
Characterization of Functional Receptors for Gastrointestinal Hormones on Human Colon Cancer Cells


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