Somatostatin binding in human gastrointestinal tissues: effect of cations and somatostatin analogues

G V Miller, S R Preston, L F Woodhouse, S M Farmery, J N Primrose

Abstract

This study characterises the somatostatin binding site in human gastrointestinal cancer and mucosa in terms of cationic specificity and relative affinity for three somatostatin analogues. Competitive displacement assays were performed on plasma membranes from human gastric and colonic tissues using radiolabelled somatostatin-14 as ligand. Comparison was made with the somatostatin binding site in rat cerebral cortex. In gastrointestinal tissue, magnesium decreased and sodium increased specific binding. By contrast, in rat cerebral cortex, the converse cationic effect was seen. These changes resulted from alterations in receptor density, with no change in receptor affinity. Displacement studies were then performed with somatostatin-14 and somatostatin analogues RC-160, somatuline, and octreotide. RC-160 and somatuline displaced radiolabel from binding sites in gastric and colonic cancer and mucosa with 10-fold lower affinity than the native peptide. Octreotide did not displace radioligand in gastric or colonic cancer at any concentration tested. By contrast, in rat cortex, although all three analogues displaced with a lower affinity than the native peptide, there was no difference between analogues. These data suggest a distinct somatostatin receptor subtype in gastrointestinal tissues.

Somatostatin is a potent inhibitory peptide that exists naturally in at least two forms. Originally described as the ‘somatotropin release inhibitory factor’ (SRIF), which is now known as somatostatin-14. Within the central nervous system somatostatin-14 and somatostatin-28 act as neurotransmitters and are influential in the regulation of sleep, motor activity, and limbic system activity. Outside of this system, somatostatin has a number of regulatory activities. In the gastrointestinal tract it inhibits endocrine and exocrine secretion, decreases smooth muscle contractility and reduces mesenteric blood flow and absorption of water, amino acids, and triglycerides. A further effect is as a natural anti-proliferative agent and recent studies have suggested that longacting somatostatin analogues may be of value in the treatment of a variety of malignancies.

It now seems that there is a family of receptors that bind somatostatin in different tissues and recently two such receptors have been cloned and sequenced. The best characterised binding sites are the high affinity somatostatin receptors in rodent cerebral cortex. These seem to exist in at least two different forms, which may be differentiated by their relative affinities for radiolabelled tyr-11-somatostatin-14 and the tyr-3-analogue (204–990) of octreotide, a longacting somatostatin analogue. A further receptor class in rodent adrenohypophysial plasma membranes can be distinguished by the differential competitive effects of somatostatin analogues and native somatostatin. Recent work has suggested that although high affinity receptors can occasionally be seen in gastrointestinal malignancies, the predominant binding is low affinity and high capacity, which may represent a further receptor subtype.

Further characterisation of somatostatin receptor subtypes has been achieved by the recognition that the ionic milieu may effect binding of somatostatin to its receptor. Srikant and colleagues have shown that monovalent (sodium and potassium) cations reduce the specific binding of [¹²⁵I]-tyr-11-somatostatin-14 to rat cerebral cortex plasma membranes in a dose dependent manner. By contrast, divalent cations such as magnesium and calcium increase somatostatin binding in rat cerebral cortex.

Because of the finding of the specific binding of somatostatin to gastrointestinal cancer plasma membranes and the documented anti-proliferative nature of somatostatin, it would seem a logical step to evaluate somatostatin as a new therapeutic agent in the treatment of gastrointestinal cancer. The use of somatostatin in gastrointestinal malignancies has been limited, however, by the short biological half life of the native peptide. In recent years this limitation has been overcome by the synthesis of a number of longacting, stable somatostatin analogues. These analogues have been applied clinically as anti-secretory agents in various conditions and are potentially of great interest to the gastrointestinal oncologist.

Therefore, the aim of this study was to characterise the low affinity somatostatin binding site in normal and malignant human gastrointestinal tissues in respect of its response to changes in cationic environment and the binding affinities of three stable somatostatin analogues. Results of these studies may allow assessment of the potential application of somatostatin analogues in treatment for gastrointestinal solid organ tumours.

Patients and methods

COLLECTION OF GASTRIC AND COLONIC CARCINOMA SAMPLES AND RAT CEREBRAL CORTEX

Twenty one patients having laparotomy for resection of gastric or colonic adenocarcinoma were included in the study (11 gastric adenocarcinoma; 10 colonic adenocarcinoma). Fresh resection specimens were opened longitudinally...
and washed with ice cold saline. Samples of mucosa from the gastric corpus and colon were dissected from the underlying muscularis layer, finely divided, and washed again in ice cold saline. Incisional biopsy specimens of the tumour (approximately 1 g wet tissue weight) were also removed and similarly finely divided and washed. All samples were snap frozen in liquid nitrogen and stored at −70°C until assayed. Female Wistar rats were killed by cervical dislocation and the brain removed. Cerebral cortex was dissected and finely divided, then snap frozen in liquid nitrogen and stored at −70°C until assayed.

PREPARATION OF PLASMA MEMBRANES

Plasma membranes were prepared using a modification of the method of Srikant and Patel. In brief, frozen stored tissue was mechanically pulvuirised and homogenised on ice in homogenising buffer (Sucrose 250 mM, KCl 25 mM, TRIS 50 mM; pH 7.4) at 10,000 rpm in short bursts for two minutes using an Ultraturrax T25 homogeniser (Scientific Instruments Ltd, Liverpool, UK). The homogenate was centrifuged at 4°C and 270 g for 10 minutes to remove nuclear debris and the supernatant removed. This supernatant represented a crude membrane preparation, which was ultracentrifuged at 4°C for 10 minutes at 15,000 g using a Beckmann L5 65B Ultracentrifuge (Beckman Laboratory Instruments Ltd, High Wycombe, Bucks, UK). The pellet was resuspended in 10 mM TRIS-HCl buffer (pH 7.4) and ultracentrifuged at 4°C for 10 minutes at 15,000 g. The final pellet was resuspended in 2 ml of 10 mM TRIS-HCl buffer (pH 7.4) and the protein concentration determined using the method of Bradford.

PREPARATION OF [125I]-TYR-11-SOMATOSTATIN-14

Iodination of tyr-11-somatostatin-14 (Sigma Ltd, UK) was performed using the chloramine T method. The iodination reaction mixture was eluted on a G-25 Superfine Sephadex column using an elution buffer of 0.1 M acetic acid with 1 mg/ml bovine serum albumin. The somatostatin containing fractions were collected and the final specific activity of the [125I]-tyr-11-somatostatin-14 was calculated at between 330 and 500 Ci/mmol.

ASSESSMENT OF CATIONIC EFFECT

Competitive displacement assays were performed upon plasma membrane preparations of rat cerebral cortex, gastric mucosa, and gastric carcinoma. The incubation buffer (HEPES, KOH 50 mM, bovine serum albumin 1-0%, bacitracin 0-01%; pH 7.4) contained different concentrations of NaCl (0–160 mM) or MgCl₂ (0–10 mM), or both. The incubation reaction (volume = 100 µl) consisted of incubation buffer, plasma membrane preparation (protein concentration 0.2 mg/ml) and 100,000 cpm of radio-labelled (approximately 2 nM), Specific binding was defined as that displaced by 10 µM somatostatin-14 (Sigma Ltd, UK). Displacement curves were constructed using somatostatin-14 as competitor over the concentration range 10⁻¹⁰ M to 10⁻¹ M. After a one hour incubation at 30°C, the reaction was stopped by the addition of 0.5 ml ice cold saline, and the bound fraction collected by centrifugation at 13,000 g for two minutes in a Microcentaur microcentrifuge (MSE Ltd, UK). The supernatant was removed by suction and the pellet washed once with 0.5 ml ice cold saline. The pellet was counted in the reaction tube on a Packard Cobra II Autogammacounter (Canberra Packard, Pangbourne, Berks, UK). All assay points were performed in triplicate and the coefficient of variation of each triplicate was <10%.

DISPLACEMENT BY SOMATOSTATIN ANALOOGUES

Under the same experimental conditions as used previously in both rat cerebral cortex and human gastrointestinal tissue plasma membranes, displacement assays were performed using long-acting somatostatin analogues as competitor. Binding assays in gastrointestinal tissues were carried out in the presence of 80 mM NaCl. Somatuline (IPSEN Pharmaceuticals, France), octreotide (Sandoz Ltd, Finsley Park, UK), and RC-160 (Debiopharm SA, Lausanne, Switzerland) were used over the concentration range 10⁻¹¹ to 10⁻¹ M. Non-specific binding was taken as the remaining bound counts after displacement with 10⁻¹ M native peptide.

DATA ANALYSIS

Receptor binding data were analysed using the Ligand PC Curve Fitting Program of Munson and Rodbard²⁰ to determine binding characteristics of native somatostatin in terms of binding affinity (Kd, nM) and maximum binding capacity (Bmax, pmol/mg membrane protein).

Displacement data from experiments in the presence of differing cationic concentration were

**Figure 1: Effect of increasing concentration of Na⁺ on specific binding to plasma membranes from human gastric mucosa (O) and rat cerebral cortex (C). Values are mean (SEM). Statistical significance for rat and human tissue, compared with values in the absence of cations is shown (*p<0.05; **p<0.01; ***p<0.001).**
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Table I: Characterisation of somatostatin binding in rat cerebral cortex and human gastrointestinal tissues

<table>
<thead>
<tr>
<th>Rat cortex</th>
<th>Gastric mucosa</th>
<th>Gastric cancer</th>
<th>Colonic mucosa</th>
<th>Colonic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀* (nM)</td>
<td>1-4 (0-2)</td>
<td>516 (75)</td>
<td>196 (56)</td>
<td>252 (40)</td>
</tr>
<tr>
<td>KD (nM)</td>
<td>0-85 (0-1)</td>
<td>623 (118)</td>
<td>241 (39)</td>
<td>271 (37)</td>
</tr>
<tr>
<td>Bmax (pmol/mg protein)</td>
<td>0-22 (0-1)</td>
<td>30-9 (5-7)</td>
<td>10-5 (4-9)</td>
<td>14-0 (3-7)</td>
</tr>
</tbody>
</table>

Values are mean (SEM); *IC₅₀ is the concentration that causes 50% inhibition; KD is the concentration of ligand which causes a 50% displacement of radiolabel; Bmax is maximum binding capacity; Binding affinity = KD/Bmax. Values of binding affinity are expressed as the percentage of total, non-specific, and specific binding occurring in the same tissues in the absence of cations. Dose response curves in the presence of Na⁺ alone, Mg²⁺ alone or a combination of increasing concentrations of one cation and a fixed concentration of the other were constructed from displacement data. To assess the kinetics of cation induced changes in receptor affinity and binding capacity, competitive displacement assays were performed in duplicate using incubation buffer containing either 120 mM Na⁺ or 10 mM Mg²⁺. From displacement data, receptor affinity (KD) and maximum binding capacity (Bmax) were calculated using Scatchard analysis. Statistical analysis of binding data was performed using non-parametric tests (Mann-Whitney U test and Wilcoxon signed rank test).

Results

Specific [¹²⁵I]-Tyr-11-Somatostatin-14 binding in rat cerebral cortex and human gastrointestinal tissues

Rapid, specific binding of [¹²⁵I]-Tyr-11-somatostatin-14 occurred in plasma membranes of rat cerebral cortex in a time and temperature dependent manner. Specific binding reached equilibrium after 30 minutes and remained stable for up to two hours. Binding assays were routinely incubated at 30°C for 60 minutes. Binding was also dependent upon protein concentration of the membrane preparation, being linear over the range of protein concentration from 0-1–2-0 mg/ml, and assays were routinely performed at a protein concentration of 0-2–0-5 mg/ml. In these studies, five rat cerebral cortex samples were examined for specific somatostatin binding sites. All samples were shown to exhibit high affinity receptors of median (range) Kd 0-85 (0-55–0-99) nM and median (range) Bmax 0-22 (0-12–0-32) pmol/mg protein. Table I shows these data, which are consistent with previous reports.²₁

Displaceable binding occurred in nine of 11 gastric and eight of 10 colonic samples (approximately 70–80% of samples examined). Sufficient material was available in six of nine gastric and six of eight colonic binding site positive cases for further studies to be performed. Somatostatin binding in both gastric and colonic tissues (‘normal’ and malignant) was seen to be of low affinity and high capacity (Table I). Binding affinity in gastric adenocarcinoma was about two to threefold higher than in gastric mucosa, although there was no difference in binding affinity when comparing colonic mucosa and tumour. The receptor density was higher in gastric mucosa, but the other tissues differed little.

Cationic influence on somatostatin binding

Over the concentration range 80–160 mM Na⁺, there was a significant (p<0-01) reduction in specific binding to rat cerebral cortex membranes to 48% of that seen in the absence of cations. By contrast with rat cerebral cortex, the addition of Na⁺ to membrane preparations from gastrointestinal tissues increased the specific binding to 130–165% of that seen in the absence of cations (p<0-001). This increase was seen over the concentration range 60–160 mM. Figure I shows these data.

In rat cerebral cortex, addition of magnesium resulted in a modest increase in specific binding reaching 135% of that in the absence of cations at a concentration of 3-75 mM (p<0-05). Increasing the [Mg²⁺] further had no effect on specific binding. In gastrointestinal tissues, the effect of Mg²⁺ appeared erratic but the addition of Mg²⁺ 10 mM reduced specific binding by 30% (p<0-05).

To determine the effects of Na⁺ and Mg²⁺ in combination, increasing concentrations of NaCl were added to incubation buffers containing 5 mM MgCl₂, and, conversely, increasing concentrations of MgCl₂ were added to incubation buffers containing 120 mM NaCl. In the presence of Mg²⁺, low concentrations of Na⁺ (20–80 mM) reduced specific binding in rat cortex to the same extent as seen in the absence of Mg²⁺. Higher concentrations of Na⁺, however, reduced specific binding to a lesser extent than if Mg²⁺ was absent (p<0-01). Thus, the deleterious effect of Na⁺ on specific binding is partially abrogated in the presence of Mg²⁺. Increasing the concentration of Mg²⁺ in the presence 120 mM Na⁺ resulted in an increase in specific binding, which mirrored that seen with Mg²⁺ alone.

In the presence of 5 mM Mg²⁺, increasing the concentration of Na⁺ had no effect on specific binding in gastrointestinal tissues, in consideration with the findings made in the absence of magnesium. This difference was statistically significant (p<0-005; Wilcoxon). Increasing the concentration of Mg²⁺ in the presence of 120 mM Na⁺ reduced the specific binding, mirroring the findings in the presence of...
Mg\(^{2+}\) alone. This effect was maximal with a Mg\(^{2+}\) concentration of 10 mM, when specific binding fell to 75% of that in the absence of cations.

Binding data analysis shows that changes in specific binding of the high affinity somatostatin receptor in rat cerebral cortex are brought about by an effect upon receptor binding capacity with there being no effect upon receptor affinity (data now shown). This agrees with the findings of Reubi and Maurer.4 In gastric mucosa, low affinity binding sites were identified in the presence of 120 mM Na\(^+\) with a Kd of 425 (170–615) nM and Bmax of 19.4 (7.9–38.8) pmol/mg protein. In the presence of 5 mM Mg\(^{2+}\), low affinity sites with a Kd of 253 (193–355) nM and Bmax of 10.7 (4.8–15.3) pmol/mg protein were seen. Statistical analysis showed a significant (p<0.02) fall in the receptor density in the presence of Mg\(^{2+}\) from that in the presence of Na\(^+\) with no significant change in receptor affinity. Similarly, for gastric carcinoma, in the presence of 120 mM Na\(^+\), low affinity sites with a Kd of 262 (175–328) nM and Bmax of 11.5 (9.1–13.2) pmol/mg protein were identified. Incubation in the presence of 10 mM Mg\(^{2+}\) showed low affinity sites with a Kd of 222 (169–265) nM and Bmax of 5.9 (3.4–6.3) pmol/mg protein. The presence of Mg\(^{2+}\) produced a statistically significant fall (p<0.02) in receptor number when compared with binding in the presence of Na\(^+\) and had no significant effect on receptor affinity (Fig 2).

**Figure 2:** Scatchard plot of binding data from human gastric cancer in the presence of 120 mM Na\(^+\) (■) and 10 mM Mg\(^{2+}\) (+). Shown is a representative experiment on the tissue from one patient. B/F=bound/free.

**Figure 3:** Displacement of \[^{[125]}\text{I}-\text{tyr-11-somatostatin-14}\] by native somatostatin-14 (○), somatuline (△), RC-160 (□), and octreotide (●) in rat cerebral cortex. Values are mean (SEM) percentage specific binding from six tissue samples (in triplicate) at each concentration tested.

**Figure 4:** Displacement of \[^{[125]}\text{I}-\text{tyr-11-somatostatin-14}\] by native somatostatin-14 (○), somatuline (△), RC-160 (□), and octreotide (●) in (A) human gastric adenocarcinoma and (B) human colonic adenocarcinoma. Values are mean (SEM) percentage specific binding from six tissue samples (in triplicate) at each concentration tested.

**DISPLACEMENT OF \[^{[125]}\text{I}-\text{TYR-11-SOMATOSTATIN-14}\] BY SOMATOSTATIN ANALOGUES**

In rat cerebral cortex all three analogues studied displaced radiolabel in competitive assays (Fig 3) although the displacement curves were shifted considerably to the right. Somatuline and...
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RC-160 displaced with comparable IC\textsubscript{50} values (about 400-fold lower affinity than native somatostatin), while octreotide displaced with about 2000-fold lower affinity than the native peptide (Table II).

Table II shows somatuline and RC-160 displaced radiolabel with IC\textsubscript{50} values in gastric carcinoma. Maximal displacement occurred at 10\textsuperscript{-4}M for both peptides with mean (SEM) displacement of 76.7-7% (9.1) and 79.8-8% (7.5) respectively of specific binding (compared with a maximal 100% displacement with somatostatin-14). Octreotide (10\textsuperscript{-5}M) maximally displaced only 47.3-3% (10-3) of specific binding, however, there being insufficient displacement to calculate an IC\textsubscript{50}. Fig 4A gives the mean displacement data expressed graphically.

In gastric mucosa, somatuline and RC-160 displaced with similar IC\textsubscript{50} values to those in gastric adenocarcinoma (see Table II). Maximal reduction (10\textsuperscript{-3}M) of specific binding was appreciably lower, however, than that occurring in gastric adenocarcinoma (59-2% (4-3) and 47% (6-7) respectively). Octreotide produced insufficient displacement to calculate an IC\textsubscript{50} with only 45-4% (8-2) mean maximal displacement at 10\textsuperscript{-4}M only 14-2% (6-3) was seen.

The findings in colonic adenocarcinoma for somatuline and RC-160 were remarkably similar to those seen in gastric adenocarcinoma (IC\textsubscript{50} values shown in Table II). Maximal reduction in specific binding occurred at 10\textsuperscript{-3}M with 87-5% (6-7) and 95-5% (3-2) displacement of radiolabel compared with the native peptide. Again, octreotide produced insufficient displacement to calculate an IC\textsubscript{50} with only 45-4% (8-2) mean maximal displacement at 10\textsuperscript{-4}M analogue concentration. Fig 4B gives the displacement data expressed graphically.

In colonic mucosa, somatuline and RC-160 displaced radiolabel to a greater degree than in gastric mucosa (maximal 74-8% (12-8) and 85-8% (5-4) displacement at 10\textsuperscript{-3}M). Octreotide (10\textsuperscript{-5}M) displaced 45-3% (9-2) of specific binding, again preventing the calculation of an IC\textsubscript{50}.

Discussion
The identification of somatostatin receptor subtypes in gastrointestinal tissues and their full characterisation, especially in terms of the binding affinities of stable analogues, is important in assessing the potential of these agents in the treatment of gastrointestinal malignancy. This is particularly the case if the anti-proliferative effect of somatostatin is a direct one. Analogues that have potent anti-secretory effects on endocrine cells by specific receptor subtypes may not be particularly active as anti-proliferative agents in tissues that predominantly express a different binding site. The potential for endocrine treatment of advanced gastrointestinal malignancy is, however, enormous. These malignancies have a poor prognosis and respond only incompletely to intensive chemotherapy. They are common and, hence, even a modest benefit in terms of survival, similar to that found with endocrine manipulations in breast cancer, would be an important advance.

This study confirms previous reports showing that high affinity somatostatin binding is present in rat cerebral cortex, and that this binding is increased by magnesium and reduced by sodium. Binding data now suggest that these changes are brought about through a change in receptor density with no significant change in receptor affinity. In human gastrointestinal tissues, a different pattern of somatostatin binding is seen. At these low affinity, high capacity binding sites the converse cationic effect seems to apply: specific binding is increased in the presence of sodium and reduced by magnesium. Further, the increased specific binding noted in the presence of sodium is reduced when magnesium is added to the system. In common with the situation in rat cerebral cortex, there is no significant change in the receptor affinity, the increased binding being brought about by increased receptor density. Taken together these data suggest that the low affinity binding seen in gastrointestinal tissues entails a different receptor subtype from the high affinity binding site in rat cerebral cortex. In this regard the somatostatin analogues investigated in the present study (Table II) do not significantly displace [\textsuperscript{111}Tyr\textsuperscript{-11}]somatostatin-14 from any of the samples tested and indeed mean maximal displacement at 10\textsuperscript{-4}M of only 14-2% (6-3) was seen.

All somatostatin analogues tested competed at the high affinity somatostatin binding site in rat cerebral cortex, albeit with a lower affinity than the native peptide. Similarly in gastrointestinal tissues, we saw a 'right shift' in the displacement curves with somatostatin analogues as competitors compared with the native peptide. There was also an appreciable difference in the binding affinities of the three analogues, with RC-160 and somatuline having similar potencies but octreotide displacing the native peptide only in very few tumours, and then only at very high concentrations. This is despite the fact that the anti-secretory effects of the three analogues are very similar.\textsuperscript{1,2} We have, however, previously shown that different somatostatin analogues have disparate effects on the growth of human gastrointestinal cell lines in vitro\textsuperscript{3} and have supported the view of Schally and co-workers that minor modifications to the basic structure of somatostatin analogues may have a profound effect upon clinical efficacy in terms of antitumour activity.\textsuperscript{2} RC-160 and somatuline have very similar structures, and this may explain why they differ so little in binding studies.

The fact that octreotide binds so poorly to tumours and that the analogue was used in such small doses may explain the lack of clinical efficacy found in some small clinical studies in patients with gastrointestinal cancer.\textsuperscript{4} Analogues such as octreotide have been more successful in the treatment of patients with malignant neuroendocrine tumours (carcinoid and VIPoma), where they inhibit tumour cell production of peptide hormones, thereby controlling the distressing paraneoplastic symptoms associated with these conditions. These tumours often express high affinity receptors to which octreotide binds. There are anecdotal reports of tumour regression on treatment with somatostatin analogues\textsuperscript{5} although the mechanism remains unclear. The effect may be mediated either by interaction with receptors on the
tumour cells or, alternatively, by inhibition of the production of local growth factors (for example, epidermal growth factor or the insulin like growth factors I and II or of growth hormone itself), which is known to stimulate local growth factor production.

The low affinity somatostatin binding, which we have found does not seem to be an artifact of the experimental method, a possibility that must be considered as the assay is performed on tissue that it enzymatically active and, hence, could degrade the ligand. We are, in fact, able to show high affinity binding sites in non-endocrine cancers from the gastrointestinal tract with this experimental method. Unfortunately, tumours of this type are uncommon and we have had insufficient tissue to investigate the effects of cations and stable analogues on specific binding at this high affinity site. In practical terms, however, high affinity binding is so uncommon that if somatostatin analogues only had activity in tumours expressing this receptor, there would be little clinical application.

Whether somatostatin analogues have an effect in gastrointestinal cancer by specific cell surface receptors on cancer cells or by inhibiting the production of local growth factors, it is clear that clinical studies should be performed in patients with gastrointestinal cancer. As regards the first postulated mechanism, it has not yet been shown conclusively that the low affinity binding site is functionally active, although experiments on the growth of cancer cell lines suggest that this is the case. It is clear, however, that studies on patients with gastrointestinal cancer should be performed using doses of appropriate analogues sufficient to produce a pharmacologically significant effect at this binding site. These studies are now in progress.

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