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Tyrosine modification increases the affinity of gastrin for ferric ions

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Abstract

The peptide hormone gastrin₁₇, which occurs naturally in both tyrosine sulphated and unsulphated forms, binds two ferric ions with pM affinities. The aim of this study was to investigate the hypothesis that sulphation or phosphorylation of gastrin₁₇ altered ferric ion binding, and/or affinity for the CCK1 or CCK2 receptor. To investigate the effect of tyrosine modification on ferric ion binding, the changes in absorbance of gastrin₁₇, gastrin₁₇SO₄ and gastrin₁₇PO₄ on addition of Fe³⁺ ions were monitored. Binding of gastrin₁₇, gastrin₁₇SO₄ and gastrin₁₇PO₄ to the human CCK1 and CCK2 receptors was assessed by competition with [¹²⁵I]-Bolton and Hunter-labelled cholecystokinin₈ in transiently transfected COS cells. Tyrosine sulphation or phosphorylation increased the affinity of gastrin₁₇ for the first ferric ion bound from 267 to 83 pM and 14 pM, respectively, but had no effect on the stoichiometry of ferric ion binding. In contrast the affinity of gastrin₁₇ for the second ferric ion bound was reduced from 94 pM to 7.32 μM and 671 nM, respectively. While sulphation of gastrin₁₇ increased its affinity for the CCK2 receptor approximately 50 fold, phosphorylation had no effect on receptor binding. These results demonstrate that tyrosine modification may have profound effects on the interaction of gastrins with ferric ions and with the CCK2 receptor.

Keywords: Gastrin, Ferric, Iron, Phosphorylation, Sulphation

Background

The classical gastrointestinal hormone gastrin₁₇ (ZGPWLEEEEEEAYGWMDFamide, Gamide) was first recognized by its ability to stimulate gastric acid secretion (Dockray et al. 2001). Human gastrin is initially synthesized as a 101 amino acid prohormone, which is processed via the 80 amino acid prohormone progastrin, to various non-amidated precursors including glycine-extended gastrin₁₇ (ZGPWLEEEEEEAYGWMDFG, Ggly) (Dockray et al. 2001). While Gamide stimulates proliferation in the gastric mucosa (Koh et al. 1999), progastrin and Ggly are important growth factors for the colorectal mucosa (Aly et al. 2004). The related hormone cholecystokinin (CCK), which has the same amidated C-terminal pentapeptide sequence as gastrin₁₇, is responsible for gallbladder contraction and pancreatic enzyme secretion (Miyasaka and Funakoshi 2003).

The receptors for CCK (CCK1 receptor) and Gamide (CCK2 receptor) are both members of the seven transmembrane domain family, and share 50 % sequence identity (Aly et al. 2004; Shulkes and Baldwin 1997). The CCK1 receptor is found on pancreatic acini and has a 1000-fold higher affinity for sulphated CCK₈ (CCK₈SO₄) than for unsulphated CCK₈. However, sulphation of CCK₈ does not greatly affect its affinity for the CCK2 receptor, which is found in the gastric mucosa and in the brain (Shulkes and Baldwin 1997). Neither the CCK1 nor the CCK2 receptor recognizes non-amidated forms of CCK or gastrin, but the receptors can be readily distinguished with several selective antagonists (Shulkes and Baldwin 1997).

Gastrins such as Ggly and Gamide bind two ferric ions (Baldwin et al. 2001), the first to Glu7 and the second to Glu8 and Glu9 (Baldwin et al. 2015; Pannequin et al. 2002). Ferric ions are essential for the biological activity of Ggly as a stimulant of cell proliferation and migration (Pannequin et al. 2002), but are not required for the biological activity of Gamide (Pannequin et al. 2004). For example, treatment with the iron chelator

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desferrioxamine, or substitution of Glu7 with Ala, completely blocked the biological activity of Ggly (Pannequin et al. 2002), but had no effect on the biological activity of Gamide (Pannequin et al. 2004).

Sulphation of CCK₈ on the sole tyrosine residue greatly increases receptor binding and biological potency (Jensen et al. 1981). Tyrosine sulphation or phosphorylation of CCK₈ increased the stoichiometry of ferric ion binding from 1 to 2, without greatly affecting the affinity (Baldwin et al. 2008). Gastrins also occur in sulphated and unsulphated forms (Dockray et al. 2001), and the sulphated form predominates in the fetal pancreas (Brand et al. 1984). Gastrin can also be phosphorylated by the EGF receptor tyrosine kinase *in vitro* (Baldwin et al. 1983), but phosphorylated gastrins have not been reported to occur naturally. The aim of the present study was to determine whether or not sulphation or phosphorylation of gastrin₁₇ altered ferric ion binding or affinity for the CCK1 or CCK2 receptor. Since non-amidated forms of gastrin require ferric ions for activity, a change in affinity for ferric ions on modification of the tyrosine residue of gastrins might have profound effects on their biological activity.

Methods

Peptides

Gastrin₁₇ and sulphated gastrin₁₇ (95 and 96 % pure, respectively) were purchased from Bachem (Bubendorf, Switzerland). Phosphorylated gastrin₁₇ (85 % pure) was from Mimotopes (Clayton, Australia). All peptides had a pyroglutamyl residue at their N-terminus and were C-terminally amidated. The impurities consisted of water and salts.

Absorption spectroscopy

The absorption of peptides (10 μM in 10 mM sodium acetate (pH 4.0) containing 100 mM NaCl and 0.005 % Tween 20) at 280 nm in the presence of increasing concentrations of ferric ions from 2.5 to 50 μM was measured against a buffer blank, in 1 ml quartz cuvettes thermostatted at 298 K, with a Cary 5 spectrophotometer (Varian, Mulgrave, Australia).

Receptor binding assay

Binding of ligands to the human CCK1 or CCK2 receptors on transiently transfected COS-7 cells was measured by competition with sulfated [¹²⁵I]-Bolton and Hunter labelled-CCK₈ (50,000 cpm/well, Amersham Biosciences, Castle Hill, Australia) as described previously (Baldwin et al. 2008).

Curve fitting and statistics

Data [expressed as mean ± standard error of the means (SEM)] for the binding of ferric ions to gastrins were

fitted to a two-site ordered model with the program BioEqs (Royer 1993; Royer et al. 1990). Receptor binding data were analyzed by one-way analysis of variance, followed by Bonferroni's *t* test. Differences with *P* values <0.05 were considered significant.

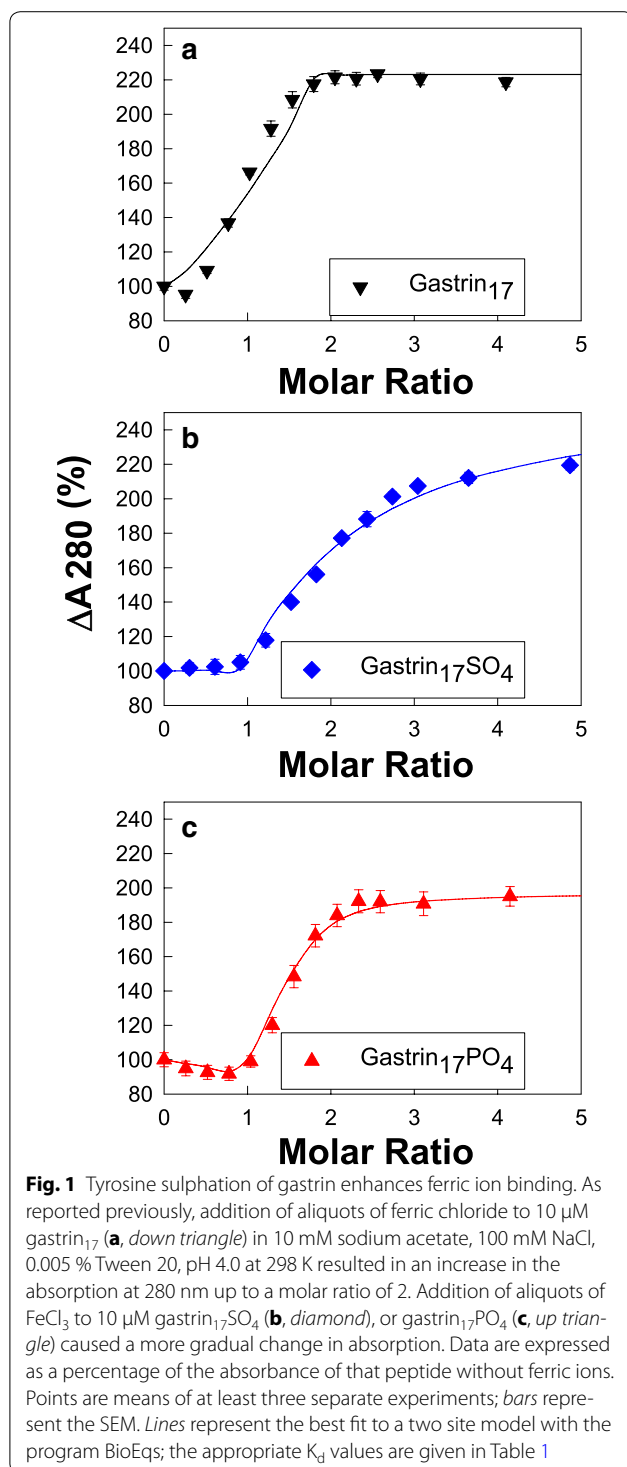
Results and discussion

Changes in absorbance on binding of ferric ions to tyrosine-modified gastrin₁₇

The effect of addition of Fe³⁺ ions on the absorption spectrum of gastrin₁₇, gastrin₁₇SO₄ and gastrin₁₇PO₄ was first investigated by absorption spectroscopy. A pH value of 4.0 was chosen in order to avoid any problems with precipitation of ferric hydroxides, and to allow direct comparison with previous studies. As reported previously (Baldwin et al. 2001, 2015) the absorption of gastrin₁₇ at 280 nm increased to a maximum of 220 % after the addition of 2.0 mol ferric chloride/mol peptide (Fig. 1a). Fitting of the absorption data to a two-site ordered model with the program BioEqs gave values for K_{d1} and K_{d2} of 267 and 94 pM, respectively, in good agreement with the values determined previously (Baldwin et al. 2015) (Table 1). The increase in absorption of gastrin₁₇SO₄ (Fig. 1b) on addition of ferric ions was more gradual than for gastrin₁₇, and in the case of gastrin₁₇PO₄ a slight decrease in absorption was initially observed (Fig. 1c). Nevertheless the absorption for both gastrin₁₇SO₄ and gastrin₁₇PO₄ finally reached maxima of 195 % to 220 % after the addition of 2–3 mol ferric chloride/mol peptide. Fitting of the absorption data to a two-site ordered model with the program BioEqs gave values for K_{d1} and K_{d2} of 83 pM and 7.32 μM, respectively, for gastrin₁₇SO₄, and of 14 pM and 671 nM, respectively, for gastrin₁₇PO₄ (Table 1).

This data indicates that, although tyrosine sulphation or phosphorylation had no effect on the stoichiometry of ferric ion binding, the affinity of gastrin for the first ferric ion was enhanced, and for the second ferric ion was reduced, by modification of the tyrosine side chain. The possibility that the hydroxyl group of the tyrosine is itself a ligand for the second ferric ion does not appear to be likely as XAFS data suggested that three of the five glutamate side chains act as ferric ion ligands (Baldwin et al. 2015). A more likely explanation is that the bulky sulphate and phosphate groups occupy the ferric ion binding site and prevent access of more than one ferric ion.

The concentrations of gastrins and of ferric ions used in the above experiments were supra-physiological. The concentration of gastrins was chosen as the minimum concentration that gave a reproducible change in absorbance on addition of ferric ions. Nevertheless our previous publications indicate that the binding of ferric ions to gastrin at *physiological* concentrations is essential for



biological function. Thus mutation of the glutamate residues essential for binding ferric ions abrogates the biological activity of Ggly as a stimulant of cell proliferation and migration (Pannequin et al. 2002). Similarly removal of ferric ions with the iron chelator desferrioxamine

Table 1 Binding of ferric ions by gastrin₁₇, CCK₈ and their derivatives

	Reference	Absorption			
		K_d1 (pM)	K_d2 (pM)	A_{280} Site 1 (%)	A_{280} Site 2 (%)
Gastrin ₁₇		267	94	100	223
Gastrin ₁₇	Baldwin et al. (2015)	300	85	100	313
Gastrin ₁₇ SO_4		83	7,320,000	88	259
Gastrin ₁₇ PO_4		14	671,000	98	215
CCK ₈	Baldwin et al. (2008)	Not detected			
CCK ₈ SO_4	Baldwin et al. (2008)	Not detected			
CCK ₈ PO_4	Baldwin et al. (2008)	Cooperative	Cooperative		194 ^a

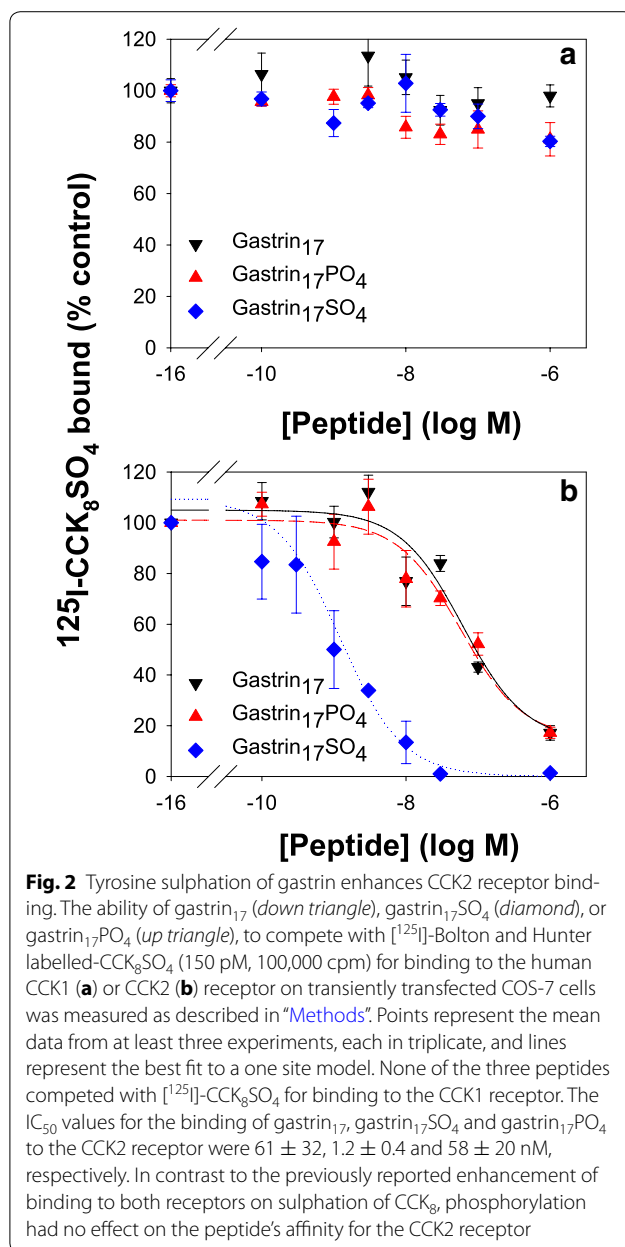
The affinity of, and the percentage absorbance change at 280 nm on, the binding of the first and second ferric ions to gastrin₁₇, gastrin₁₇ SO_4 or gastrin₁₇ PO_4 at pH 4.0 were determined by fitting the mean data obtained in the absorbance experiments (N = 3) described in the Fig. 1 legend to a two site ordered model with the program BioEqs

^a The absorbance changes for CCK₈ and its derivatives were measured at 275 nm as the peptide contains tyrosine and phenylalanine, but no tryptophan, residues (Baldwin et al. 2008)

(Ferrand et al. 2010; Pannequin et al. 2002), or by competition with bismuth ions (Kovac et al. 2012; Pannequin et al. 2002), blocked the biological activity of Ggly in vitro and in vivo.

Effect of tyrosine modification on CCK receptor binding

The effect of tyrosine modification on the binding of gastrin₁₇ to either human CCK1 or CCK2 receptors was then examined. Although sulphation of gastrin₁₇ increased its affinity for the human CCK2 receptor, phosphorylation of gastrin₁₇ had no effect on CCK2 receptor binding (Fig. 2b). The IC_{50} values for the binding of gastrin₁₇, gastrin₁₇ SO_4 and gastrin₁₇ PO_4 to the CCK2 receptor were 61 ± 32 , 1.2 ± 0.4 and 58 ± 20 nM, respectively. The IC_{50} values for the binding of gastrin₁₇ were slightly higher than the values, which range from 0.94 to 6.4 nM, previously reported for the cloned human CCK2 receptor (Ito et al. 1993; Lee et al. 1993; Pisegna et al. 1992). The affinity of gastrin₁₇ SO_4 for the cloned human CCK2 receptor does not appear to have been reported previously, but data for binding to dispersed gastric glands from the guinea pig fundus also suggest that sulphation increases the affinity for the CCK2 receptor, in that case by a factor of tenfold, from 16 to 1.6 nM (Praisman et al. 1983). Binding of gastrin₁₇, gastrin₁₇ SO_4 and gastrin₁₇ PO_4 to the CCK1 receptor was not detected in our experiments (Fig. 2a). In the case of gastrin₁₇ this result agrees with the data of de Weerth and co-workers, who reported



an IC₅₀ value of 1.8 μM (de Weerth et al. 1993); binding affinities of gastrin₁₇SO₄ and gastrin₁₇PO₄ for the CCK1 receptor do not appear to have been reported previously.

Comparison with CCK₈

In contrast to CCK₈, where tyrosine sulphation or phosphorylation increased the stoichiometry of ferric ion binding from 1 to 2 without greatly affecting the affinity (Baldwin et al. 2008), tyrosine sulphation or phosphorylation of gastrin₁₇ had no effect on the stoichiometry of ferric ion binding, but enhanced the affinity of gastrin₁₇ for the first ferric ion, and reduced the affinity for the

second ferric ion. This difference is not unexpected as the gastrin binding site consists of three of the five glutamate residues, while the CCK₈SO₄ binding site involves the two aspartate residues, and the CCK₈PO₄ binding site the phosphate group itself (Baldwin et al. 2008). In terms of receptor binding, tyrosine sulphation enhanced the affinity, while phosphorylation reduced the affinity, of CCK₈ for both the CCK1 and CCK2 receptors (Baldwin et al. 2008). In contrast, while tyrosine sulphation enhanced the affinity of gastrin₁₇ for the CCK2 receptor, phosphorylation had no effect. Although the generalization that modification of tyrosine profoundly affects the binding of ferric ions is valid, the differences between two such closely related peptides as gastrin₁₇ and CCK₈ indicate that the exact binding details will have to be determined for each peptide individually.

Conclusions

While tyrosine sulphation or phosphorylation of gastrin₁₇ had no effect on the stoichiometry of Fe³⁺ ion binding, the affinity for the first ferric ion bound was increased from 267 to 83 pM and 14 pM, respectively. Sulphation of gastrin₁₇ increased its affinity for the CCK2 receptor approximately 50 fold, but phosphorylation had no effect on receptor binding. These results imply that tyrosine modification may have profound effects on the biological activities of gastrins.

Abbreviations

CCK: cholecystokinin; DMEM: Dulbecco's Modified Eagle Medium; Gamide: amidated gastrin₁₇; Ggly: glycine-extended gastrin₁₇.

Authors' contributions

GSB conceived the study, conducted the absorbance experiments, and drafted the manuscript. IS participated in the design of the study and performed the binding experiments. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests. The research did not involve animals or human participants.

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References

- Aly A, Shulkes A, Baldwin GS (2004) Gastrins, cholecystokinins and gastrointestinal cancer. *Biochim Biophys Acta* 1704:1–10
- Baldwin GS, Knesel J, Monckton JM (1983) Phosphorylation of gastrin-17 by epidermal growth factor-stimulated tyrosine kinase. *Nature* 301:435–437
- Baldwin GS, Curtain CC, Sawyer WH (2001) Selective, high-affinity binding of ferric ions by glycine-extended gastrin(17). *Biochemistry* 40:10741–10746

- Baldwin GS, Bailey MF, Shehan BP, Sims I, Norton RS (2008) Tyrosine modification enhances metal-ion binding. *Biochem J* 416:77–84
- Baldwin GS, George GN, Pushie MJ (2015) High Affinity Binding of Indium and Ruthenium Ions by Gastrins. *PLoS one* 10:e0140126. doi:10.1371/journal.pone.0140126
- Brand SJ, Andersen BN, Rehfeld JF (1984) Complete tyrosine-O-sulphation of gastrin in neonatal rat pancreas. *Nature* 309:456–458
- de Weerth A, Pisegna JR, Huppi K, Wank SA (1993) Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. *Biochem Biophys Res Commun* 194:811–818
- Dockray GJ, Varro A, Dimaline R, Wang T (2001) The gastrins: their production and biological activities. *Annu Rev Physiol* 63:119–139
- Ferrand A, Lachal S, Bramante G, Kovac S, Shulkes A, Baldwin GS (2010) Stimulation of proliferation in the colorectal mucosa by gastrin precursors is blocked by desferrioxamine. *Am J Physiol Gastrointest Liver Physiol* 299:G220–227
- Ito M et al (1993) Functional characterization of a human brain cholecystokinin-B receptor. A trophic effect of cholecystokinin and gastrin. *J Biol Chem* 268:18300–18305
- Jensen SL, Holst JJ, Nielsen OV, Rehfeld JF (1981) Effect of sulfation of CCK-8 on its stimulation of the endocrine and exocrine secretion from the isolated perfused porcine pancreas. *Digestion* 22:305–309
- Koh TJ, Dockray GJ, Varro A, Cahill RJ, Dangler CA, Fox JG, Wang TC (1999) Overexpression of glycine-extended gastrin in transgenic mice results in increased colonic proliferation. *J Clin Invest* 103:1119–1126
- Kovac S, Loh SW, Lachal S, Shulkes A, Baldwin GS (2012) Bismuth ions inhibit the biological activity of non-amidated gastrins in vivo. *Biochem Pharmacol* 83:524–530
- Lee YM, Beinborn M, McBride EW, Lu M, Kolakowski LF Jr, Kopin AS (1993) The human brain cholecystokinin-B/gastrin receptor. Cloning and characterization. *J Biol Chem* 268:8164–8169
- Miyasaka K, Funakoshi A (2003) Cholecystokinin and cholecystokinin receptors. *J Gastroenterol* 38:1–13
- Pannequin J, Barnham KJ, Hollande F, Shulkes A, Norton RS, Baldwin GS (2002) Ferric ions are essential for the biological activity of the hormone glycine-extended gastrin. *J Biol Chem* 277:48602–48609
- Pannequin J, Tantiongco JP, Kovac S, Shulkes A, Baldwin GS (2004) Divergent roles for ferric ions in the biological activity of amidated and non-amidated gastrins. *J Endocrinol* 181:315–325
- Pisegna JR, de Weerth A, Huppi K, Wank SA (1992) Molecular cloning of the human brain and gastric cholecystokinin receptor: structure, functional expression and chromosomal localization. *Biochem Biophys Res Commun* 189:296–303
- Praissman M, Walden ME, Pellecchia C (1983) Identification and characterization of a specific receptor for cholecystokinin on isolated fundic glands from guinea pig gastric mucosa using a biologically active 125I-CCK-8 probe. *J Recept Res* 3:647–665
- Royer CA (1993) Improvements in the numerical analysis of thermodynamic data from biomolecular complexes. *Anal Biochem* 210:91–97
- Royer CA, Smith WR, Beechem JM (1990) Analysis of binding in macromolecular complexes: a generalized numerical approach. *Anal Biochem* 191:287–294
- Shulkes A, Baldwin GS (1997) Biology of gut cholecystokinin and gastrin receptors. *Clin Exp Pharmacol Physiol* 24:209–216

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