BENZON SYMPOSIUM No. 53

THE NEW BIOLOGY OF THE GASTRIN-CHOLECYSTOKININ FAMILY OF HORMONES

OCTOBER 9-12, 2006, COPENHAGEN, DENMARK

Organizing committee:
Jens F. Rehfeld (Copenhagen), Jens Bundgaard (Copenhagen), Anders H. Johnsen (Copenhagen), Rolf Håkanson (Lund) and Arne Svejgaard (Copenhagen)

Abstracts - MONDAY, October 9, 2006

INVENTION OF GASTRIN RECEPTOR ANTAGONISTS

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The invention of new drugs, artefacts, is always based on antecedent discoveries in physiology and biochemistry.

The invention of potent and selective gastrin antagonists followed on, first, from the discoveries that circulating gastrin was a 17mer polypeptide and, more important, that the entire efficacy in the hormone was contained in the terminal amidated tetrapeptide. However, the invented peptidic antagonists lacked oral bioavailability and were eventually shown to be partial agonists that were fully efficacious in the highly geared ECL cells in conscious dogs.

The second discovery was that Aspergillus alliaceous broth contained an alkaloid that blocked CCK receptors. Merck chemists then elucidated the structure of the complex alkaloid, asperlicin. They 'cut out' and developed a benzodiazepine motif. Later, chemists at Lilly developed a quinazolone motif. These and other research groups invented many highly potent and selective gastrin receptor antagonists.

Several compounds have been evaluated in human Phase 1 studies but none of them have been developed clinically.

The third discovery was that the gastrin tetrapeptide was structured as a 3:10 helix that brought the aromatic rings of tryptophan and phenylalanine close together. Using this information, JBF chemists eventually invented a series of new gastrin receptor antagonists. The results of the use of one of these compounds to treat pancreatic cancer will be discussed.

AN OVERVIEW OF NEUROENDOCRINE PRECURSOR PROCESSING

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One of the unique features of eukaryotic organisms is the presence of a highly organized series of organellar structures which together constitute the secretory pathway, allowing for the biosynthesis, intracellular transport and maturation of a large number of biologically important secreted peptides and proteins. In the case of peptide hormones and neuropeptides, maturation often involves chemical modifications such as amidation, acetylation, acylation, glycosylation, sulfation or phosphorylation, but another important aspect is the proteolytic processing of larger, less active or inactive, precursors to release one or more biologically active peptides. Recent years have brought great advances in our understanding of the nature of the enzymes involved in the proteolytic maturation of precursor proteins and their sites of action within the secretory pathway.

The facility of the major neuroendocrine subtilisin-like proprotein convertases PC1/3 and PC2, along with others such as PC5/6 and furin, in differentially processing larger polyfunctional precursors of varying complexity to generate appropriate mixtures of product peptides appears to be an important evolutionarily conserved feature of their design.

PROPROTEIN CONVERTASES AND THEIR INHIBITORS

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The conversion of neuroendocrine precursors into bioactive peptides proceeds via sequential enzymatic steps which begin with cleavage by serine proteinases known as prohormone convertases. Our research has focused on the biochemistry and cell biology of these interesting enzymes. We have shown that PC1 is quickly activated early in its biosynthesis and generates an 87 kDa species which is then further cleaved to a more active but unstable smaller protein. We have used combinatorial peptide library screening to show that both of these enzymes are potently inhibited by the peptide LLRVKR, a sequence within a neuroendocrine protein known as proSAAS. ProPC2, on the other hand, interacts with its binding protein 7B2 early in its biosynthesis and proceeds to the later stages of the secretory pathway as a zymogen in complex with this protein. In the secretory granules, proPC2 undergoes pH-dependent cleavage to its active form. Mutagenesis studies have shown that when the active site serine is mutated to alanine, the mutant proPC2 is efficiently synthesized and secreted in 7B2-expressing cells, providing a potential avenue for production of crystallizable quantities of a convertase zymogen form. In addition to the domain which binds to proPC2, 7B2 also contains a C-terminal sequence which represents a potent inhibitor of this enzyme, similarly to proSAAS. We are continuing to search for both natural and synthetic inhibitors of various convertase family members.

Our recent work is directed towards a molecular understanding of cleavage site specificity via mutagenesis of convertase binding pockets as well as developing *in vitro* posttranslational modifying systems. We have now generated an *in vitro* system for bioactive peptide synthesis using the known precursors proopiomelanocortin, proenkephalin, and proghrelin coupled with recombinant processing enzymes. Our results demonstrate that our *in vitro* system can generate mature peptides from each precursor in reasonable yields. We are currently optimizing recoveries, using novel precursors to generalize our results, testing other posttranslational modifying enzymes, and preparing samples for cell-based bioactivity assays.

BIOGENESIS AND DELIVERY OF SECRETORY GRANULES

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Neuroendocrine secretory granules (SGs) are formed at the *trans*-Golgi network as immature SGs (ISGs). Subsequently, they undergo maturation characterized by acidification and condensation of the lumen, processing of cargo proteins and removal of proteins in small clathrin coated vesicles. We selectively monitored ISGs in neuroendocrine PC12 cells by expressing human chromograin B fused variants of GFP. This showed that ISGs were transported within seconds to the F-actin rich cortex, where they matured and exhibited myosin Va-dependent movements. Co-expression of a dominant negative mutant of myosin Va (MCLT) revealed that the restriction of SGs in the F-actin rich cortex was strongly reduced and the removal of the endoprotease furin, which normally takes place within 30 minutes after biogenesis of ISGs, was blocked. Notably, similar effects were observed when mutants of rab 3 isoforms were co-expressed in PC12 cells. In summary our results suggest that both myosin Va and rab 3 isoforms are involved in the remodelling of the SG membrane during maturation.

NEURONAL CCK SYNTHESIS

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CCK is synthesized as a precursor protein which undergoes limited proteolysis, removal of carboxyl terminal arginine residues and carboxyl-terminal amidation to generate biologically active CCK. The most likely candidates for the endoproteolytic enzymes are the prohormone convertases (PCs), particularly PC1, PC2 and PC5. We have shown that they are widely co-localized with CCK in rat brain. Our analysis of PC1 and 2 knockout (ko) mice show that they are essential for CCK processing in some regions of mouse brain. However, in the absence of each of these enzymes, CCK levels are never zero so other endoproteases are acting.

The possible role of cathepsin L (CL), a newly identified prohormone processing enzyme which cleaves pro CCK on the amino terminal side of the basic residue was analyzed in CL ko mice and in CCK-expressing AtT-20 cells. CCK levels in cerebral cortex from CL ko mice were reduced compared to wild-type while PC1 ko mice were the same as wild type and PC2 ko mice were actually higher than wild type.

Treatment of CCK expressing AtT-20 cells with the CL-specific inhibitor CLIK148 decreased CCK secretion. Novel CCK products ArgCCK8 and LysCCK22 which represent CL cleavage products of

proCCK were found in media from these cells and co-eluted on HPLC with their appropriate synthetic peptides. Inhibition of CL with CLIK 148 caused a decrease in the ratio of these forms to CCK8 and CCK 22, while media from AtT-20 cells expressing PC1 RNAi had an increased ratio.
The talk will review our knowledge of the biosynthesis and processing of CCK in endocrine cells and protease ko mice and describe recent results that support the hypothesis there two independent pathways consisting of multiple PC enzymes and CL paired with an aminopeptidase that work in parallel to insure the production of active CCK.

IDENTIFICATION OF TYROSINE SULFATION BY MASS SPECTROMETRY

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Tyrosine sulfation is a posttranslational modification found in receptors as well as in secreted proteins including peptide hormones. The sulfated tyrosine is typically involved in protein-protein interaction or provides increased stability against proteases. To date only about 60 proteins are known to be tyrosine sulfated though the number of proteins carrying the modification is belived to be substantially higher. In chemokine receptor CCR5, a co-receptor for HIV, sulfation of tyrosine is essential for HIV entry. This serves as an example to how important it is to expand on our knowledge of tyrosine sulfation. The tyrosine sulfate is relatively unstable in low pH as well as during mass spectrometry, and there is no sequence independent tyrosine sulfate specific antibody available, thus analysis is difficult. The aim of this study was to develop a method for identification of tyrosine sulfated residues from complex samples. This was done by a mass spectrometry based method that exploits the lability of the sulfate in the mass spectrometer for specific identification. Alternating between slightly elevated and lowered collision energies in a Q-TOF instrument, a neutral loss of 80 Da was used as a diagnostic marker for sulfated tyrosine. Phosphorylated tyrosine gives rise to a similar neutral loss but the tyrosine phosphate is much more stable and requires higher collision energy. Furthermore phosphorylated tyrosine gives a stable immonium ion in MSMS spectra, which can be used to distinguish between the two. The result is a mass spectrometric technique for specific identification of new sulfation targets in mixtures.

SORTING OF PROGASTRIN AND PROCCK

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The peptide prohormones, progastrin and procholecystokinin, undergo extensive intracellular posttranslational processing during intracellular transport and storage in secretory granules prior to subsequent release upon external stimulation. The processing includes tyrosine O-sulfation, endoproteolytic cleavage and carboxyterminal amidation. The latter is a prerequisite for the acid-secretion stimulatory effect of gastrin, and occurs in the regulated secretory granules and transport of progastrin derivatives to this cell organelle is therefore necessary for normal function of the G-cell.
We have examined the requirements for correct sorting of progastrin to the regulated secretory granules. We show that endoproteolytic cleavage at N-terminal residues initiate in early compartments of the secretory pathway, and that the N-terminal part of progastrin is not necessary for sorting to the regulated granules and concluding bioactivation. In contrast, progastrin sorting depends on two types of "sorting signals": the di-basic residue motifs constituting the endoproteolytic cleavage sites, and an acid motif located within gastrin-17. The sorting elements act in synergy, which suggests that they are acting sequentially during sorting. Further studies of tyrosine O-sulfation of progastrin processing products and progastrin mutants allows us to propose a two-step sorting model of progastrin to the regulated secretory granules. The model suggests that different derivatives of progastrin can be more or less specifically secreted by either of three ways: constitutively, constitutive-like or regulated.
Considering the homology between gastrin and CCK, we have searched for signals in proCCK necessary for sorting to the regulated pathway based on our findings in progastrin. We find that proCCK is sorted by basic residue motifs, but in contrast to progastrin, disruption of basic residue motifs alone can lead to constitutive secretion. Hence, proCCK may depend only on basic residues for sorting.

EXTRACEREBROINTESTINAL CCK SYNTHESIS

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Cholecystokinin (CCK) expression is a feature of the brain and the gut. In the brain, CCK acts as a neurotransmitter whereas intestinal expression harbours endocrine CCK. More recently, the muscular heart

has also emerged as an endocrine organ that secretes potent natriuretic peptides. Atrial myocytes display structural and biochemical features resembling specialized endocrine cells, and the enzyme responsible for amidation of gastrin and cholecystokinin (PAM) is abundantly expressed in the cardiac myocytes. We therefore hypothesized that the heart may be capable of gastrin or CCK synthesis. Our findings in normal porcine heart so far are: 1) Normal, atrial tissue contains proCCK in concentrations comparable to that of cardiac natriuretic peptides. In contrast, ventricular tissue contains proCCK but no natriuretic peptides. 2) Only minor amounts of amidated CCK is present in heart extracts, and gel filtrations suggest a larger proCCK form. 3) Preliminary experiments indicate that cardiac CCK gene expression can be rapidly stimulated through activation of adrenergic receptors. 4) No gastrin mRNA or peptide expression is detectable in any of the cardiac regions. Our findings have several implications. Firstly, the endocrine heart expresses not only natriuretic peptides but also the CCK gene. Moreover, there are major differences between cellular storage of natriuretic peptides and proCCK in the cardiac regions. Finally, despite the abundant presence of PAM, cardiac proCCK seems to elute amidation. The heart is therefore is a CCK expressing organ that can shed new light on cellular proCCK synthesis and maturation.

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GENETIC DISSECTION OF GASTRIN AND CCK PATHWAYS THAT CONTROL GASTRIC ACID SECRETION

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Analysis of knockout (KO) mice, including KO of genes encoding gastrin, CCK, gastrin receptor, histidine decarboxylase (HDC), H_2 receptor, M_3 receptor, and somatostatin$_2$ (SST_2) receptor, may provide insight into the mechanisms that regulate gastric acid secretion. In wild-type (WT) mice, gastrin stimulates the parietal cells by mobilizing histamine from ECL cells. In addition, muscarinic stimulation contributes to the acid response. Acid secretion was impaired in gastrin KO and even more so in gastrin receptor KO mice, probably because of few and inactive ECL and parietal cells. In HDC KO mice, parietal cells responded to histamine but not to gastrin. H_2 receptor KO mice failed to respond to either gastrin or histamine. In gastrin/CCK double KO mice, a poor acid response to gastrin was associated with low HDC in ECL cells. However, acid was produced in response to vagal stimulation (pylorus ligation) and to histamine. CCK-8 inhibited the acid response to pylorus ligation and to histamine while increasing somatostatin mRNA expression in the oxyntic mucosa. Carbachol stimulated the acid response to pylorus ligation in WT mice but reduced it in gastrin/CCK double KO mice. M_3 receptor KO mice failed to respond with acid secretion to deoxy-glucose (a vagal stimulant) and responded poorly to both gastrin and histamine. In H_2 receptor KO mice, there was no acid response to gastrin and the response to carbachol was poor. In SST_2 receptor KO mice, the acid response to gastrin was enhanced but not that to histamine. Thus, the gastrin-histamine pathway is coordinated with the CCK-somatostatin pathway in regulating acid secretion. In the parietal cells, the H_2 receptor interacts with the M_3 receptor and the SST_2 receptor with the CCK_2 receptor.

ENDOCRINE FORMS AND NOVEL PHYSIOLOGY OF CHOLECYSTOKININ

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The presence of CCK-58 in tissue and in the circulation has been reported in multiple mammalian species including man. Different endocrine amounts of CCK-58, CCK-33, CCK-22, and CCK-8 have been reported by various laboratories. This variability was very evident in endocrine forms of cholecystokinin in rat. A new RAPID method (Rapid processing, Acidified blood, Protease inhibited, Ice temperatures, and Diluted blood) has been developed for processing blood. This method improves recovery and eliminates degradation of radiolabeled CCK-58 and several other radiobabeled endocrine peptides. Using the RAPID method, only CCK-58 was detected in rat blood after stimulation with camostat or casein. The synthesis of sulfated CCK-58 has allowed us to compare its physiology with CCK-8. CCK-58 differs markedly from CCK-8 for patterns of pancreatic secretion in vitro, ability to stimulate pancreatic water secretion in vivo,

composition of electrolytes in the secretion in vivo, induction of pancreatitis, and inhibition of food intake. Our data support the hypothesis that pancreatic fluid secretion as well as protein secretion is regulated by exogenous CCK-58, independently of secretin. CCK-58 is also an abundant molecular form of cholecystokinin in the brain, where it has qualitative differences in actions from CCK-8. The biologically active regions of CCK-58 and CCK-8 have identical primary structures, but different solution structures. We propose this difference in tertiary structure accounts for the difference in biological actions between CCK-8 and CCK-58. We continue to test the hypothesis that CCK-58 is the only endocrine form of cholecystokinin and that the physiology of CCK-58 differs markedly from the agonist most often used to study cholecystokinin physiology, CCK-8.

DROSOPHILA AS A MODEL SYSTEM FOR STUDYING CHOLECYSTOKININ/GASTRIN RECEPTOR MEDIATED PHYSIOLOGY

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Bioinformatic analysis of the Drosophila genome revealed the presence of two candidate cholecystokinin/gastrin like receptors (CG32540 and CG6857). These receptors (DSKR1 and DSKR2) were cloned, expressed and pharmacologically characterized in our laboratory. The genes encode 584 and 532 amino acid proteins respectively. Comparison of each fly GPCR with the human CCK-1 and CCK-2 receptors reveals overall amino acid identities of 37-40%. When expressed *in vitro*, both receptors are fully activated by the Drosophila cholecystokinin-like peptides, drosulfakinin 1 and 2. Like their mammalian counterparts, both fly GPCRs showed higher potency for the sulfated (vs. non-sulfated) forms of endogenous ligands. Neither fly receptor recognized mammalian CCK octapeptide or gastrin despite conservation of 8 of 11 pocket residues that have previously been shown to confer ligand affinity to the mammalian CCK-2R. It will be of interest to further explore the molecular basis of this divergence. *In vivo* studies demonstrated that both fly receptors are expressed in the head and body of Drosophila. In addition, transcripts for these proteins can be detected during development (i.e. in larvae and in pupae). To enable assessment of receptor mediated physiology with potential parallels to CCK/gastrin mediated function in vertebrates, we have established a series of sensitive *in vivo* assays. These include quantification of food intake, assessment of intestinal motility, and detection of acid production in larval Drosophila. Utilizing a transgenic RNA interference approach, we have generated flies in which the DSKR2 transcript has been targeted. Behavioral analysis of these flies will be presented.

PRODUCTION, SECRETION AND BIOLOGICAL ACTIVITY OF PROGASTRIN DERIVED PEPTIDES

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The assumption that amidated gastrin (Gamide) - the final processed product - is the only bioactive form is no longer tenable because of the finding that progastrin (proG) and other non-amidated intermediates are not inert but are able to stimulate proliferation, potentiate gastric acid secretion and promote the development of colorectal carcinoma (CRC). Since the biological activity of the gastrin intermediate gastrin-gly (Ggly) and proG was first described, there has been intense interest in the physio- and pathophysiologic roles of these peptides. However it is not generally appreciated that they constitute relatively small proportions of the final gastrin products in the antrum and in most gastrinomas and CRC. We have analysed using region specific radioimmunoassays the relative amounts of proG, Gamide, Ggly and the C-terminal flanking peptide of proG (CTFP) in the antrum from normal subjects, in resected CRC and in the circulation. The bioactivity of CTFP *in vitro* was assessed. Similar studies on the production and secretion of proG and proG intermediates in sheep were performed.

The identity of the antral CTFP was confirmed as the hexapeptide progastrin$_{75\text{-}80}$ by HPLC and mass spectroscopy. The CTFP hexapeptide is by far the major stored form of progastrin in antrum (CTFP 8.7;Gamide 2.0 nmol/g), resected CRC (CTFP 19; Gamide 0.07 pmol/g) and plasma (CTFP 800; Gamide 13 pmol/l). Similarly in sheep, the CTFP was the major antral and secreted form of gastrin. Contrary to

previous suggestions, CTFP is biologically active (proliferation, migration, signal transduction) with a similar potency to Ggly.
In summary, the CTFP is the major stored and secreted form of progastrin-derived peptides in the antrum and circulation of normal subjects and in the tumours of patients with CRC. Taken together with the findings that CTFP is biologically active, we suggest that CTFP is not an inactive metabolite of progastrin processing but a bioactive peptide with potential roles in the normal and diseased gastrointestinal tract.

SIGNAL TRANSDUCTION PATHWAYS MEDIATING THE ANTI-APOPTOTIC ACTIONS OF GASTRIN IN PANCREATIC CANCER CELLS

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We reported that G17 has growth promoting and anti-apoptotic effects on the AR4-2J pancreatic adenocarcinoma cell line (Am. J. Physiol.273:G891-G898, 1997 and Am. J. Physiol. 280: G298-G307, 2001). In particular, we observed that while MAPK regulates G17-stimulation of AR4-2J cells proliferation, Akt mediates the anti-apoptotic action of G17. The clinical significance of these findings has been underscored by the observation that inhibition of the proliferative and anti-apoptotic actions of gastrin in the pancreatic cancer cell line PAN1, leads to increased sensitivity of the cells to the action of cytotoxic agents (Gastroenterology. 122: A241, 2002). Accordingly, we examined the signal transduction pathways mediating G17 stimulation of AR4-2J cells growth and survival. G17 activated the small GTP binding proteins Ras, Rac, Rho and Cdc42. Transduction of the cells with adenoviral vectors expressing dom. neg. Akt, Ras, Rho and Cdc42, but not dom. neg. Rac, inhibited AR4-2J cells proliferation and survival. Both exoenzyme C3 from Clostridium Botulinum (C3), a toxin known to inactivate Rho, and PD98059, a MAPK inhibitor, reversed G17 inhibition of AR4-2J cells apoptosis. G17 induction of Akt activation was reduced by more then 60% by both dom. neg. Ras and Rho and by 30% by dom. neg. Cdc42. In contrast, G17-stimulated MAPK activation was blocked by more then 80% by dom. neg. Ras, but not by dom. neg. Rho and Cdc42. Similar results were observed in the presence of C3. Dom. neg. Rac failed to affect G17 induction of both Akt and MAPK while it inhibited by almost 50% sorbitol- but not-G17-stimulated activation of p38 kinase. Akt phosphorylates and inactivates pro-apoptotic molecules such as BAD and the FOXO transcription factors, forkhead/winged-helix nuclear proteins, which are known to regulate the transcription of pro-apoptotic genes. Thus, we studied the effect of G17 on the phosphorylation of both BAD and FOXO3 using western blots with anti-phospho-BAD and -FOXO3 antibodies. G17 induced the phosphorylation of both BAD and FOXO3 in the AR4-2J cells. We examined if gastrin induced phosphorylation of FOXO transcription factors leads to inhibition of FOXO transcriptional activity. For these experiments, the AR4-2J cells were transfected with plasmids expressing FOXO1 together with a vector containing three copies of the insulin-responsive sequence of the insulin-like growth factor binding protein 1 promoter placed upstream of the luciferase reporter gene (3xIRS-Luc). FOXO1 induced 3xIRS-Luc transcriptional activity and G17 inhibited this effect by 30%. In summary, G17 promotes AR4-2J cells growth and survival through the activation of multiple GTP binding proteins, which in turn, regulate different protein kinase cascades. While Ras activates Akt and MAPK, Rho and Cdc42 appear to regulate Akt and possibly other, yet unidentified, kinases mediating the growth stimulatory actions of G17. In addition, G17 stimulates the phosphorylation of FOXO transcription factors and it inhibits their transcriptional activation. These findings underscore the complexity of the signal transduction pathways mediating the anti-apoptotic actions of gastrin in pancreatic cancer cells and they might provide new clues for a better understanding of the mechanisms that control pancreatic cancer growth .

CHOLECYSTOKININ (CCK) REDUCES FOOD INTAKE THROUGH A CENTRAL, AND NOT A MYENTERIC PATHWAY

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Our laboratory focuses on examining the role of the myenteric plexus of the gut, in the reduction of food intake by CCK. We have reported the following data in adult Sprague Dawley rats. Firstly, exogenous CCK-8 reduces food intake and increases Fos-like immunoreactivity (Fos-LI), a marker for neuronal activation, in the myenteric plexus of the duodenum and jejunum and in food intake regulatory areas of the

dorsal vagal complex (DVC). Secondly, devazepide, a specific CCK_1 receptor antagonist inhibited Fos-LI appearance. Thirdly, subdiaphragmatic vagotomy abolished only DVC Fos-LI caused by exogenous CCK-8. Fourthly, chemical sympathectomy, by daily injections of guanethidine sulfate for five weeks, attenuated, but did not abolish Fos-LI in the myenteric plexus caused by exogenous CCK-8. In addition, exogenous CCK-8 failed to increase Fos-LI in the following locations: celiac and cranial mesenteric ganglia, dorsal root ganglia from the fourth thoracic (T4) to the second lumbar (L2) vertebrae, and spinal cord segments L4-L2. Finally, recently we found that endogenous cholecystokinin, released in response to orogastric gavage of the non-nutrient, trypsin inhibitor camostat mesilate (200 mg/kg), reduced food intake and increased Fos-LI only in the DVC. These actions were attenuated by CCK_1 receptor blocker and vagotomy. Unlike CCK-8, camostat failed to increase Fos-LI in the myenteric neurons of the duodenum and jejunum. Collectively, these results demonstrate that unlike exogenous CCK-8, endogenous cholecystokinin reduces food intake and increases Fos-LI by central actions, which do not involve the myenteric plexus. Furthermore, a previous report indicated that CCK-58 is the only form of cholecystokinin released in response to camostat, and the only endocrine form in rats. This raises the possibility that CCK-58 differs in its actions from CCK-8 for stimulation of Fos-LI in the myenteric plexus, but not for stimulation of central Fos-LI.

TYROSINE PHOSPHATASE SHP-2 IN CCK2 RECEPTOR SIGNALLING: ROLE OF AN ITIM-LIKE MOTIF

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SHP-2, a non receptor tyrosine phosphatase has been shown to transduce mitogenic and pro-migratory signals from different RTK. Since the CCK2 receptor is now recognized to mediate the mitogenic effects of gastrin on gastrointestinal and pancreatic cells, we analyzed the potential role of SHP-2 in CCK2R signalling and the molecular mechanisms involved.

The tyrosine residues Y390 and Y438 within the CCK2R sequence belong to ITIM-like motifs and represent potential tyrosine phosphorylation sites.

Pull down assays with peptides corresponding to the CCK2R ITIM-like sequences, phosphorylated or not on the tyrosine residues, performed in cell lines expressing the CCK2R show an interaction of SHP-2 with the phosphorylated Y438.

By SPR analysis (Biacore 3000) we confirmed the direct interaction between the SH2 domains of SHP-2 and the phosphorylated Y438 of the CCK2R. In COS-7 cells transfected with the wild type CCK2R (WT-CCK2R), we observe a time-dependent increase in the amount of SHP-2 co-precipitated with the receptor in response to gastrin. This increased CCK2R/SHP-2 binding after gastrin treatment is not observed when the Y438 is mutated into phenylalanine (Y438F-CCK2R). Our results also show that the Y438F-CCK2R mutant can not mediate gastrin-stimulated AKT activation in contrast to the WT-CCK2R and that inhibition of SHP-2 also blocks this activation.

SHP-2 has been shown to undergo phosphorylation on tyrosine residues. Besides an adapter role for SH2 containing proteins, phosphorylation of SHP-2 may contribute to positively regulate its phosphatase activity. In response to gastrin we observe an increase in the phosphorylation of SHP-2 with the WT-CCK2R whereas the mutation Y438F blocks this response.

In addition, we show in vivo that the targeted CCK2R expression in mice pancreas of Elas-CCK2 mice, leads to an increase in the tyrosine phosphorylated form of SHP-2.

TACHYPHYLAXIS OF THE ECL-CELL RESPONSE TO PACAP: DEPLETION OF SECRETORY PRODUCTS OR DESENSITIZATION?

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The ECL cells in the acid-producing part of the rat stomach secrete histamine and pancreastatin (PST) in response to gastrin and PACAP. Both peptides were administered to conscious rats by microinfusion into the gastric submucosa. The microdialysate was analyzed for histamine, PST and somatostatin. While PACAP raised microdialysate histamine/PST in a transient manner, the response to gastrin was sustained. It is unlikely that the transient nature of the histamine response to PACAP reflects inadequate resynthesis of histamine, since both gastrin and PACAP activated histidine decarboxylase (HDC) and since omeprazole-induced hypergastrinemia, while raising the HDC activity greatly, did not prolong the histamine response to PACAP. Unlike gastrin, PACAP mobilized somatostatin. Co-infusion of somatostatin abolished the

histamine-mobilizing effect of both PACAP and gastrin. However, pretreatment with a somatostatin receptor type-2 antagonist did not prolong the histamine response to PACAP, suggesting that endogenous somatostatin does not bring about the cessation of the response to PACAP. Repeated pulse administration of PACAP (1 h infusions with 1 h intervals) failed to induce a second histamine response. Pretreatment with a low dose of PACAP abolished the response to a subsequent near-maximal PACAP challenge. The response to gastrin was however not impeded by a PACAP pulse, nor was the the response to PACAP affected by a VIP pulse.
The transient nature of the histamine/PST response to PACAP may reflect either desensitization of the PACAP receptor or exhaustion of a PACAP-specific storage compartment.

PROGASTRIN AND COLON CANCER

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Accumulating evidence has provided a strong link between progastrin-derived peptides and colorectal cancer. Gastrin gene expression is frequently upregulated in human colorectal cancer, with significant production of progastrin (PG) and other incompletely processed peptides. Work from our laboratory has shown that the gastrin gene is a direct downstream target of the Wnt (Apc/ beta-catenin) signaling pathway. More recently, we have discovered that other oncogenic/tumor suppressor pathways, such as Ras, can modulate Wnt-dependent regulation of the gastrin promoter, and this synergistic interaction appears to involve SMAD recruitment. In addition, p73, a p53 family member, can bind the gastrin promoter and upregulate gastrin transcription, supporting the notion that the gastrin gene is downstream of numerous oncogenic pathways. Support for biological activity for progastrin arose initially from murine models developed by our laboratory. Studies from our group employing gastrin over-expressing and gastrin-deficient mice have demonstrated that progastrin or glycine-extended gastrin can modulate colonic proliferation and intestinal tumorigenesis in mouse models of intestinal neoplasia, and glycine-extended gastrin promotes the growth of lung cancer. In addition, hGAS mice with elevated circulating progastrin exhibit higher levels of mitosis in the intestine after DNA damage, associated with elevated cdk4/cyclin D1, and this activity is related to the C-terminal 26 amino acids. The proliferative effects of progastrin-derived peptides are associated with an upregulation in the intestine of Src kinase along with other signaling molecules including STAT3, P13K/Akt and JAK2. The biological activity and activated signaling revealed in these *in vivo* models suggests a receptor-mediated process, and several binding candidate proteins have been identified by other laboratories. Using a sensitive non-radioactive assay in which biotinylated peptides were combined with FACS analysis allowed the detection of binding of at least 1nM PG, and we were able to demonstrate robust PG binding to several cancer cell lines of epithelial origin. Overall, the data suggest the progastrin acts directly on epithelial cells to modulate proliferation and can contribute to neoplastic progression.

PROGASTRIN AND ITS PRODUCTS: DEFINITIONS AND ACTIVITIES

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There is continued interest in progastrin, the gastrins and other progastrin products in both basic biology and pathogenetic studies (major cancers, diabetes and gastrointestinal diseases). Discussion of these studies has, however, been hampered by inconsistent and imprecise naming. Consequently, it is about time to agree on unambiguity, where progastrin means the unprocessed progastrin molecule itself, and where the cellular end product is acid-stimulatory amidated-gastrins, and flanking fragments. In between are the many processing intermediates (for instance the glycine-extended gastrins). For years growth-promoting and other effects have been suggested for progastrin, glycine-extended gastrins and now also for flanking fragments. Therefore, consensus about the criteria for being a hormonal growth-factor should also be reached. Such criteria should probably include molecular definitions of receptor mechanisms. Examples illustrating the nomenclature confusion and the need for bioactivity criteria will be presented and discussed.

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THE NEW BIOLOGY OF THE GASTRIN-CHOLECYSTOKININ FAMILY OF HORMONES

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CHOLECYSTOKININ SYSTEMS IN THE BRAIN

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Mapping of cholecystokinin (CCK) by radioimmunoassay and histochemical techniques has revealed a wide distribution of the transcript and the peptide in the rodent brain. CCK coexists with classic transmitters in several systems, including mesencephalic ascending dopamine neurons and cortico-striatal, presumably glutamaterigic neurons. CCK is also present at high levels in the mossy fibers of the dentate gyrus of the hippocampal formation, thus coexisting with the excitatory transmitter glutamate as well as with several other peptides. The expression of CCK has been shown to be regulated in a number of experimental and animal brain-disease models. In particular, seizure activity has been shown to reduce CCK expression in the granule cell-mossy fiber system, contrasting the dramatic upregulation of, for example, enkephalin, galanin and neuropeptide Y (NPY). A similar finding has also been made in mice infected with prions and in some Alzheimer mouse models. It has been suggested that upregulation of NPY and galanin serves to dampen seizure activity and to promote regeneration. It may be speculated that downregulation of CCK, which in general induces stimulatory activity, may serve to reduce the excitatory tone and thus to counteract seizure activity. Recently, we have observed that CCK levels also change in limbic brain regions during the rat estrous cycle, with the lowest concentrations in proestrous

A major challenge is the analysis of the distribution and regulation of the two CCK receptors, CCK1 and CCK2. Initial studies on the rat brain were based on autoradiographic ligand binding technique and revealed a wide distribution of CCK2 with CCK1 being more restricted. Radu, Lindefors and colleagues have analysed the distribution of CCK1 mRNA in monkey and human brain, including brains from schizophrenic patients (see D. Radu, Aspects on the psychopharmacology of cholecystokinin, Ph.D. Thesis, Karolinska Institutet, ISBN:91-7140-368-X). So far only few studies have reported the distribution of CCK1 and CCK2 receptor protein using immunohistochemsitry, probably related to well-known difficulties to raise specific and sensitive antibodies to 7-transmembrane, G-protein-coupled receptors.

CCK IN PSYCHIATRIC DISORDERS

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The involvement of cholecystokinin (CCK) in human anxiety is well documented. Exogenous administration of CCK-2 receptor (R) agonists, such as CCK-tetrapeptide (CCK-4) and pentagastrin, provoke panic attacks in human that are identical to spontaneous attacks experienced by patients with panic disorder (PD). PD patients are hypersensitive to CCK-2R stimulation compared to healthy controls and to patients with other anxiety disorders or with major depression. Moreover, the panicogenic effect of CCK-2R agonists are antagonized by antipanic treatment in PD. Mechanistically, human studies reveal that neurotransmitter systems postulated to play a role in the modulation of human anxiety, such as the GABA, beta-adrenergic and serotoninergic systems, might be mediators of CCK-2R agonist-induced panic attacks. The primary panicogenic site of action of CCK-2R agonists has not been determined. However, PET and

fMRI imaging of CCK-2R agonist-induced panic attacks in healthy volunteers reveal changes in cerebral activity in regions known to be involved in the regulation of human anxiety.
In sum, clinical studies suggest that PD might be the result of an abnormal function of the CCK system. One approach in testing this hypothesis is to determine whether CCK receptor gene variation may be associated with PD. Genetic studies in clinical populations have yielded contradictory results probably because of inclusion of patients with different degrees of illness severity and co-morbidity. Studies limiting their subject sample to those suffering from primary, predominant and moderate to severe PD reveal a relationship between polymorphic variation of the CCK-2R gene and PD. These clinical results are strengthened by results from animal studies that show a relationship between genetic variations of CCK-2R and expression of anxiety.
In conclusion, there is evidence supporting the hypothesis of an abnormal function of the CCK system in PD. Confirmation of the hypothesis would be aided by demonstration in PD of other anomalies of the CCK system and of the therapeutic efficacy of pharmacologically viable CCK-2R antagonists.

CCK AND GUT-BRAIN SIGNALING

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CCK acts at CCK-1 receptors on vagal afferent neurons to acutely inhibit food intake, delay gastric emptying and stimulate pancreatic enzyme secretion. Neurons expressing CCK-1 receptors also express the receptors for a variety of other neurohumoral factors implicated in control of feeding behaviour. Recent work suggests the expression of some of these receptors, and also of peptide transmitters, in vagal afferent neurons is controlled by CCK. Thus in rats, food restriction for 12-18 hours is associated with increased expression of the orexigenic peptide, melanin concentrating hormone (MCH), and decreased expression of the satiety peptide, cocaine and amphetamine regulated transcript (CART). In parallel, and in the same vagal afferent neurons, food restriction leads to increased expression of MCH-1 and cannabinoid (CB)-1 receptors. Refeeding of fasted rats rapidly down-regulates MCH, MCH-1 and CB-1 expression and this is attributable to endogenous cholecystokinin (CCK). Exogenous ghrelin prevents the action of CCK in down-regulating the expression of MCH, and of MCH-1 and CB-1 receptors. These observations suggest a hitherto unexplored capacity for integration of nutrient signals at the level of vagal afferent neurons and indicate modulation of these signals by changes in gene expression in response to prior food intake mediated by CCK.

ECL CELLS: OPERATIONAL CONTROL AND ANTICIPATED FUNCTION

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ECL cells occur in the vertebrate stomach. In mammals, they constitute 1-2 % of the oxyntic mucosa. They produce, store and secrete histamine, chromogranin A-derived peptides (e.g. pancreastatin) and an unidentified peptide hormone. Ultrastructurally, they display electron-lucent secretory vesicles that are rich in histamine, and a few electron-dense granules. Secretory peptides and proteins occur in the dense cores. Histamine and peptides are released concomitantly by exocytosis. The ECL cells operate under the control of circulating gastrin (stimulatory endocrine pathway), local somatostatin (inhibitory paracrine pathway) and PACAP, VIP and galanin (neurocrine pathways). In addition, prostaglandins suppress the activity. Gastrin releases histamine from the ECL cells to cause acid secretion. Whether other signals that activate the ECL cells (e.g. PACAP) cause acid secretion too is debated. Ischemia (arterial occlusion, endothelin, adrenaline) induces a spectacular, burst-like release of histamine (not pancreastatin) from the ECL cellls. The signaling cascade, initiated by anoxia and leading to histamine release, remains unexplored. The structure / function of the ECL-cell peptide hormone is unknown. Ablation of gastric endocrine cells (gastrectomy) results in osteopenia and impaired insulin secretion. A recent report has identified parathyroid hormone-related peptide as a candidate ECL-cell hormone.
Conceivably, ECL-cell hormones control bone metabolism and insulin release by stimulating Ca uptake into bone and glucose-evoked insulin release. Exploring the significance of the ECL cells may generate new treatments of osteoporosis and obesity/diabetes.

MECHANISM OF ISCHEMIA-EVOKED HISTAMINE MOBILIZATION FROM RAT STOMACH ECL CELLS

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Histamine in the stomach resides in ECL cells (and mast cells). The ECL cells release histamine and pancreastatin in response to gastrin. Gastric schemia is known to mobilize large amounts of histamine. We compared the ECL-cell response to ischemia with that induced by gastrin.

First, we studied the vasoconstrictive effects of endothelin, vasopressin, adrenaline, angiotensin II and gastrin in vitro using circular segments of branches of the rat gastric artery. Endothelin, vasopressin and adrenaline were powerful vasoconstrictors, angiotensin II and gastrin were not. Secondly, gastric submucosal microdialysis was used to monitor histamine and pancreastatin mobilization and to determine the lactate/pyruvate ratio (index of hypoxia) in response to ischemia (clamping of the gastric artery and microinfusion of vasoconstrictors). While microinfusion of gastrin caused sustained release of histamine and pancreastatin, gastric ischemia mobilized histamine but not pancreastatin) in a burst-like manner. The staining intensity of histamine-immunoreactive ECL cells was reduced, while pancreastatin immunostaining seemed unaffected. The ECL cells displayed unchanged numbers of granules and secretory vesicles, but the volume density of microvesicles was reduced. The ECL cells recovered from the ischemia after about one week.

We conclude that ischemia mobilizes histamine but not pancreastatin from the ECL cells, possibly through the release of endocytotic microvesicles.

CHOLECYSTOKININ LEVELS IN THE RAT BRAIN DURING THE ESTROUS CYCLE

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Cholecystokinin (CCK) peptides have been found to be anxiogenic after peripheral or intracerebral administration and suggested to be implicated in mood-related disorders. CCK is one of the most abundant peptides in the mammalian nervous system, the predominant molecular form in the brain being the sulphated octapeptide (CCK-8S). CCK-8S is widely distributed with the highest levels in cerebral cortex, hippocampus, striatum and amygdala. In the present we found substantial changes in CCK levels in cingulate and frontal cortex, hippocampus, striatum and hypothalamus during a normal estrous cycle in the rat. Thus, as compared to di-estrous and estrous, CCK-like immunoreactivity (LI) monitored in tissue extracts by radioimmunoassay was significantly reduced in these brain areas during pro-estrous, the phase with the highest plasma estradiol levels (average 50 – 60%). No such effect on CCK concentrations was observed in the parietal/temporal/occipital cortex. In the medulla oblongata, cerebellum and the pituitary CCK levels were barely detected. In a previous study we have shown, using similar methodology, that galanin-LI is increased in the hippocampal formation during pro-estrous to 125% (Hilke et al., Eur. J. Neurosci., 21, 2089- 2099, 2005). However, no changes in NPY levels were observed in this brain area during the cycle (Hilke, unpublished data). These results show that circulating hormones in the female rat can influence the CCK (and at least one other peptide) primarily in the limbic system, a brain circuitry implicated in emotions. This is interesting, since depression, the most common neuro-psychiatric disease, has a higher incidence for women compared to men, with a relation to estrogen deprivation.

IMMUNE ACTIVATION IN THE CENTRAL NERVOUS SYSTEM DURING NEUROPATHIC PAIN: ROLE OF CHOLECYSTOKININ B AND TOLL-LIKE 4 RECEPTORS

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The cholecystokinin B (2) receptor knockout (*Cckbr* KO) protects against neuropathic pain, a chronic pain syndrome caused by nerve damage. The mechanism of this phenomenon is unknown, but must involve persistent changes and detailed information on these changes could give us new strategies for the treatment of neuropathic pain. We performed a gene expression study in two brain areas (midbrain and medulla) in Cckbr knockout and controls, following surgical induction of a neuropathic pain syndrome (CCI).

We found a pattern of expression differences suggesting that following nerve injury, the immune system is activated in higher brain structures and that CCK may play a role in the regulation of innate immunity in the CNS. These differences include many genes related to the MAPK pathway, including the Toll-like receptor 4 (*Tlr4*). We consider up-regulated *Tlr4* giving protection to the development of neuropathic pain

in *Cckbr* deleted mice. Using microarrays, a powerful and systematic approach, we have replicated previous research implicating the MAPK pathway in the development of neuropathic pain. Furthermore, our findings suggest that following nerve injury, the immune system is activated also in higher brain structures and that *Tlr4-Cckbr* interaction may regulate the innate immunity in the CNS.

AN INTRON ONE POLYMORPHISM IN THE CHOLECYSTOKININ RECEPTOR A GENE IS ASSOCIATED WITH SCHIZOPHRENIA IN MALES

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Cholecystokinin (CCK) has been suggested to be involved in several psychiatric disorders such as panic disorder and schizophrenia. Recent studies have shown that a variation located in intron one 5 basepair upstream exon two in the cholecystokinin receptor A (CCK-AR) gene might be associated with schizophrenia.

We analyzed this polymorphism in patients from the Danish Psychiatric Biobank diagnosed with Schizophrenia (n=349), major depression (n=384), and panic disorder (n=150), and compared the frequencies to the background population (n=898). We found a higher frequency of the C allele in the schizophrenia group (f(C)=0.143) than compared to the controls (f(C)=0.116). Thus we investigated this polymorphism in a separate group of patients with schizophrenia (f(C)=0.151) and found the same tendency (p=0.008 for genotypes and p=0.019 for alleles). Interestingly, when dividing the samples according to sex, we found a highly significant difference between males with schizophrenia (f(C)=0.171) compared to control males (f(C)=0.109) (p=0.002 for genotypes and p=0.0008 for alleles). We found no difference among any of the other samples examined.

The variation is located in the intron-exon boundary and might cause an incomplete splicing. The splice efficacy was analyzed using an exon-trapping assay, and we found no indication of ineffective splicing. It is unknown whether it is the variation itself or a linked variation that causes the susceptibility to schizophrenia in males.

GASTRIN, THE ENDOCRINE PANCREAS AND DIABETES MELLITUS

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Optimal control of blood glucose homeostasis depends on an adequate number of insulin-producing beta cells in the pancreatic islets of Langerhans. The total mass of beta cells can adapt during life to meet the metabolic demands whereas failure to do so leads to the development of chronically elevated blood glucose, or diabetes. Chronic hyperglycemia is associated with a further decline in the beta cell mass and patients may become dependent on exogenous insulin treatment. This is also the case for patients suffering from autoimmune or inflammatory destruction of beta cells. However, insulin treatment does not provide a cure to the disease and can be associated with both hypoglycemic and hyperglycemic episodes leading to severe complications. Transplantation of pancreas or islet cells has proven effective to normalize glycemic control in diabetes patients but this treatment is seriously hampered by donor shortage and by the need for continuous immune suppression. Restoration of the beta cell mass through pharmaceutically induced endogenous beta cell mass regeneration represents a very attractive new therapeutic option. Gastrin is considered as a potential candidate pharmaceutical to control beta cell growth and regeneration. Although gastrin cells are not present in the adult pancreas, they are transiently found in fetal (~E15) and neonatal pancreas and also the CCK-B receptor is developmentally regulated in the pancreas, suggesting an involvement of the hormone in pancreatic development. However, gastrin -/- transgenic mice have a normal islet mass. On the other hand, hypergastrinemia can increase the regeneration of pancreatic tissue following injury in several experimental models. This was first reported in transgenic mice with pancreatic tissue injury caused by overexpression of TGF-a. We found that in rats that underwent duct ligation to cause pancreatic tissue injury, administration of gastrin leads to a rapid growth of the beta cell mass and this effect can be abrogated by a CCK-B receptor antagonist. In diabetic mice, treatment with a combination of gastrin and EGF induced a partial regeneration of the beta cell mass and was found to restore the pancreatic insulin content and normalization of glycemia. Gastrin is not a mitogen for beta cells and regeneration of the beta cell mass in these models is independent of the proliferation of pre-existing beta cells. In conditions of tissue injury, CCK-B receptor is upregulated in exocrine cells. These cells retain the potential

to transdifferentiate into endocrine islet cells. However, in an in vitro model of transdifferentiation gastrin appears unable to induce beta cell neogenesis from exocrine cells. This may indicate that gastrin acts indirectly in vivo.

FUNCTIONING OF CHOLECYSTOKININ AND GASTRIN RECEPTORS

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The cholecystokinin and gastrin receptors (CCK1R and CCK2R) are a G protein receptors which regulate a number of central and peripheral physiological functions. Activation of these receptors and/or expression of constitutively active variants may contribute to human diseases. These receptors remain potential targets to treat human diseases. The search for specific agonist and antagonists of these receptors has been an important challenge during the last few years, leading to the discovery of a set of chemically distinct compounds. However, several early antagonists have turned out to be partial agonists. Moreover, interspecies genetic polymorphism that do not alter cholecystokinin-induced signaling was shown to markedly affect activity of synthetic ligands. Our aims have been to delineate binding sites of these receptors and to gain insight into their mechanisms of activation. We have used site-directed mutagenesis, pharmacological and biological analysis of mutants and state-of-art methods of molecular modeling. We have been able to explain why JMV180, an analogue of CCK is a partial agonist on the CCK1R. More recently, we related the binding of two chemically close compounds (JB1 and JB2) differing by presence or absence of a methyl group, to their contrasting pharmacological features at the CCK2R, namely partial inverse agonist and partial agonist behavior, respectively. These data open the possibility of target-based optimization of GPCR non peptide ligands which is currently applied to a nonpeptide ligand of the CCK2R.

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GASTRIN AND CCK IN GASTROINTESTINAL CANCER

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Gastrin and CCK belong to the family of gut/brain peptides with identical carboxy-terminal pentapeptide-amide sequence. These neuropeptides exert multiple biological actions in mammalians via at least two distinct G protein-coupled receptor subtypes termed CCK1 and CCK2 receptors. The growth factor action of gastrin/CCK peptides in the pathogenesis of gastrointestinal cancer has been an area of intense research in the past decade that has provided deeper insight into tumor biology. Although trophical actions of gastrin on the gastric mucosa have been well-established, the effect of mature amidated gastrin, progastrin and intermediates to colon neoplasia in humans can still be regarded as controversial. While epidemiological evidence from patients with elevated serum gastrin levels related to pernicious anemia does not support an increased risk for colon cancer, one recent study identified that prolonged hypergastrinemia is associated with an increased risk for colon cancer in humans. The extent to which these trophical actions of gastrin in colorectal cancer in humans are mediated by functional CCK2 receptors, by CCK2 receptor splice variants, or by receptors for immature forms of gastrin which have not been cloned thus far, is an ongoing research focus. However, immunoneutralization studies using antibodies directed against the biologically active site of the peptide have already provided evidence in mice that gastrin might serve as a novel target in the treatment of colon cancer. Biological tumor therapy represents an emerging field in cancer research which may also be accessible for CCK/gastrin peptides.

GASTRIN/CCK RECEPTOR EXPRESSION IN CANCERS

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The past 2 decades have shown that peptide receptors being overexpressed in human cancers can be successfully targeted in vivo. This was originally demonstrated for overexpressed somatostatin receptors targeted in vivo with radiolabeled somatostatin analogs for diagnosis and therapy for neuroendocrine tumors. We have recently expanded this tumor targeting approach by evaluating the expression of other peptide receptors in cancers. This report focuses on CCK1 and CCK2 receptors evaluated using in vitro receptor autoradiography in several hundreds of resected human tumors using radiolabeled gastrin or CCK analogs. The results showed a particularly high incidence and density of CCK2 in non-gastrointestinal tumors, in particular in medullary thyroid carcinomas, followed by small cell lung cancers and bronchial carcinoids, but also in leiomyosarcomas, while CCK1 were found primarily in meningiomas and leiomyosarcomas. Interestingly, in gastrointestinal tumors, high incidence and density of CCK2 (and, to a lower level, of CCK1) were found mainly in gastrointestinal stromal tumors (GIST) and neuroendocrine tumors, while gastric, pancreatic and colonic carcinomas rarely expressed CCK receptors with this morphological binding method. However, contamination with CCK receptor-expressing mucosa, muscle, nerve and glands were often observed in samples containing the 3 latter cancer types.

The identification of CCK receptor-expressing tumors has triggered the development of chelated gastrin and CCK peptidic analogs for clinical application. These compounds demonstrated the feasibility to successfully target CCK2-expressing medullary thyroid carcinomas and gastrointestinal neuroendocrine

tumors for the diagnosis and subsequent radiotherapy of these tumors in patients. While the development of improved (i.e. lower kidney uptake) gastrin/CCK analogs is progressing and more tumor types (GIST, SCLC) are being evaluated for in vivo CCK receptor targeting, the available data strongly indicate that the in vivo targeting with radiolabeled CCK analogs of human cancers overexpressing CCK receptors is becoming a powerful tool in oncology.

WHY LOSS OF GASTRIN AND SONIC HEDGEHOG PRECEDES GASTRIC ATROPHY AND CANCER

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A striking aspect of sonic hedgehog (Shh) function is not only its role in the development of the gastrointestinal tract, but also its ability to maintain the differentiated gastric phenotype in the adult. Like Shh mutant mice, gastrin null mice display metaplastic changes in the stomach but also progress to gastric cancer. Since loss of Shh correlates with gastric atrophy, we hypothesized that gastrin regulates Shh levels in the parietal cell. We show here that gastrin, the hormone that stimulates acid secretion, regulates both Shh expression and processing. Gastrin treatment of canine parietal cell cultures increased secretion of the 19kDa biologically active peptide that subsequently was blocked by the proton pump inhibitor omeprazole. Using in vitro translated Shh peptide and extracts from human corpus, we demonstrated that pepsinogen A converted to pepsin A in the presence of gastrin is responsible for increased levels of the processed 19kDa form. However, in human gastric tumors, Shh processing was absent. This was due to the loss of pepsinogen A. Therefore processing of Shh in the stomach is hormonally-regulated, acid-dependent and mediated by the protease pepsin A. Loss of Shh precedes the development of atrophy in both mouse models and human examples of gastric cancer.

GASTRIC CANCER WITHOUT GASTRIN

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Gastrin deficiency and proton pump inhibitor (PPI) treatment causes achlorhydria which predispose to disease. To elucidate the underlying molecular biology we examined the changes in gastric gene expression in both types of achlorhydria. We also explored the associated changes in the gastric microflora and the long-term consequences of gastrin-deficient achlorhydria.

Expression profiles were generated from gastric RNA from wild type, gastrin knockout (KO), gastrin KO after one week of gastrin infusion, and wild type mice treated one month with a proton pump inhibitor. The results were confirmed using real time PCR and immunohistochemistry. Selective media were used to characterize the gastric microflora.

The number of gastric bacteria was increased in both gastrin KO and PPI treated mice. The expression profiles revealed activation of immune defense genes, interferon regulated response genes and intestinal metaplasia of the gastric mucosa. In young gastrin deficient mice gastrin infusions reversed the changes. Over time the changes accumulated, became irreversible and progressed into metaplasia and polyps development. Finally, the study showed that gastrin regulated the expression of genes encoding extra-cellular matrix proteins.

Independently of gastrin achlorhydria is associated with gastric bacterial overgrowth and intestinal gene expression patterns and is associated with predisposition to disease. Gastrin is therefore essential for prevention of gastric disease, mainly through control of acid secretion but to a lesser extent also through control of gastric gene expression. The gastrin deficient mouse serves as a useful new model for gastric meta- and neoplasia.

LABORATORY DIAGNOSIS OF GASTRINOMA

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Clinical diagnosis of gastrinoma remains problematic in a significant number of patients, due to other conditions associated with hypergastrinaemia, which are commonly found in patients with GI symptoms. Patients with excessive circulating gastrin include those with gastrinoma and much more commonly those where the negative feedback is absent or impaired. In addition, patients visiting outpatient clinics are not fasted.

We have collated data from routine measurement of gastrin in subjects with the following conditions- gastrinoma, auto-immune atrophic gastritis (AIAG), ideopathic gastric achlorhydria (IGA), patients on

proton pump inhibiter therapy (ppi), H-pylori (Hp) +ve duodenal ulcer (DU), Hp+ healthy controls, Hp- healthy controls after an overnight fast or post-prandially. Gastrin was assayed in plasma using an antibody (R98) directed towards the C-terminal of gastrin 17 that detects G-17 and G-34 in equimolar quantities. In addition, antisera raised to the N-terminus of G-17 and antisera raised to the N-terminus of G-34 were used. N=50 in each fasting group and N=25 in each group post-prandially. (In IGA N=25). Post-prandial specimens were collected 2 hours after a standard protein meal. Chromogranin A (CgA) was measured in selected specimens from each group.
Fasting reference range (RR) for gastrin (R98) in this laboratory is 0-45pmol/L. Results are given in pmol/L as medians (with range). In the gastrinoma group gastrin was 267 (57-31,857) giving complete overlap with AIAG, 545 (55-21,429). 12 % of gastrinoma patients presented with gastrin <70 and 24% with gastrin <200pmol/L. In IGA gastrin was 52 (2-714) (56%>RR), on ppi therapy 41 (7-262) (40%>RR), in DU Hp+ 36 (8-242) (25%>RR), in control Hp+ 24 (10-105) (14%>RR) and in control Hp- 19 (2-38) (all within RR). Use of regional specific antisera did not clarify diagnosis neither did CgA measurement.
In conclusion, diagnosis of gastrinoma is not confirmed chemically without specific additional information. As interpretation is complex, substantial experience is necessary.

FUNDIC GLAND POLYPS CAUSED BY PROTON PUMP INHIBITION ARE NOT RELATED TO HYPERGASTRINEMIA AND SERUM CHROMOGRANIN A IS AFFECTED BY MEALS

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Backgound/aims: The use of proton pump inhibitors (PPI) is associated with the development of fundic gland polyps and some patients develop multiple polyps. The use of PPI is associated with hypergastrinemia and gastrin has a well known trophic effect on the oxyntic mucosa and especially on the enterochomaffin-like (ECL) cell. It was of interest to examine whether patients developing polyps had a more pronounced gastric hypoacidity and/or hypergastrinemia. We also wanted to measure serum chromogranin A (CgA) concentrations as CgA is an ECL cell marker. Methods: Five patients having used PPI for more than one year with multiple (>10) fundic gland polyps, but a normal stomach evaluated by endoscopy when starting PPI, were included. Gender- and age-matched patients (n=6) without polyps having used PPI for a similar number of years, as well as healthy individuals (n=6) were used as controls. All PPI users underwent 24-hour gastric pH-metry and repeated measurements of gastrin and CgA in the course of one day with standardized meals, whereas only serum gastrin and CgA were measured in the healthy individuals. Helicobacter pylori status was determined in all participants.
Results: There were no significant differences in 24-hour gastric pH, serum gastrin or CgA concentrations between PPI users having developed polyps and those without polyps. All patients with polyps were H pylori negative, whereas 4 of 6 PPI users without polyps were H pylori positive. Fasting serum CgA concentrations were elevated above the upper reference value in all PPI users and more than doubled during the day. CgA concentrations in healthy individuals also doubled during the day and several subjects reached values above what is considered an upper normal value. Conclusions: Fundic gland polyps caused by proton pump inhibition are not related to hypergastrinemia. CgA in serum is affected by meals and should be measured in samples from fasting patients when used as a neuroendocrine tumour marker.

PREMALIGNANT CHANGES DEVELOPING AFTER TWO MONTHS OF HYPERGASTRINEMIA IN FEMALE JAPANESE COTTON RATS ARE REVERSED BY ANTRECTOMY

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Introduction: Inbred female cotton rats have an incidence exceeding 25% of spontaneous gastric carcinomas developing from ECL-cells. Two months of hypergastrinemia results in dysplasia and more than 4 months results in cancer in all female cotton rats. Although there are numerous animal models of gastric carcinogenesis, few have been used to study reversibility of premalignant changes during hypergastrinemia .
Material and methods: Animals with 2 months hypergastrinemia underwent antrectomy. A small wedge-shaped part of the major curvature of the oxyntic mucosa adjacent to the antrum was also removed to be able to compare the dysplastic changes at operation with the changes seen at termination of the study. Gastric pH was measured preoperatively. Age-matched normogastrinemic female animals were used as

controls. Four months after the operation the animals were sacrificed and plasma gastrin and gastric pH were measured. Mucosa thickness was measured.
Results: The mean mucosa thickness of the hypergastrinemic animals was unchanged four months after antrectomy (0.606 ± 0.09mm vs. 0.600 ± 0.013 mm). In normogastrinemic animals the mean mucosa thickness was 0.405 ± 0.04 mm before operation vs. 0.495 ± 0.06 mm at sacrifice.The plasma gastrin levels in hypergastrinemic animals were reduced from 508 ± 220 pM to 47 ± 42 pM. In control animals, mean plasma gastrin levels were 25 ± 0.4 pM vs. 31 ±18 pM at sacrifice. No macroscopic tumour growth was observed in the operated animals. Seven of eight hypergastrinemic controls had macroscopically visible tumour mass at termination of the study.
Conclusions: Antrectomy after two months hypergastrinemia prevents the development of ECL cell derived carcinoma in female cotton rats. This finding indicates that the dysplastic changes seen after two months are reversible and that further stimulation with gastrin is needed for these changes to proceed to carcinoma.

EXPRESSION OF GASTRIN IN HUMAN GASTRIC CANCER TISSUE
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Background: The expression of gastrin and its specific receptor in adenocarcinomas of the pancreas, lung, ovary and colon suggests that gastrin may act as a local growth factor and could play a role in the progression of tumor development and growth. The purpose of this study was to investigate the expression of gastrin and its receptor in human gastric carcinoma tissue.
Methods: The local expression of gastrin was measured using a library of sequence-specific radioimmunoassays for progastrin and its fragments. Both bioactive, amidated gastrins and glycine-extended processing intermediates were measured in extracts of human gastric adenocarcinomas (n=23) and their respective resection margins (n=17).
Results: In carcinomas resected from the cardia region of the stomach, amidated gastrins were present in 11 of 17 carcinomas (median 0,2 pmol/g; range 0,1-37 pmol/g) and in 8 of 11 (median 0,1 pmol/g; range <0,1-0,2 pmol/g) resection margins. Glycine-extended intermediates were found in 16 of 17 (median 0,1 pmol/g; range <0,1-5 pmol/g) carcinomas and in 5 of 11 (median 0,2; range <0,1-1,1 pmol/g) resection margins. In carcinomas of the antrum (n=3) and corpus (n=3) regions higher levels of bioactive gastrins (median 13 pmol/g, 61,1 pmol/g respectively) were detected, while the concentrations of glycine-extended intermediates (median 2,2 pmol/g, 0,9 pmol/g respectively) resembled those measured in cardia cancer tissue.
Conclusion: Our results show that only low levels of gastrin are expressed in human gastric carcinomas with origin in the cardia region, but in higher levels in adenocarcinomas from the antrum and the corpus regions. However, the findings might reflect constitutive secretion, which has been reported in other tumors. Thereby our results do not exclude that gastrin can act as a local growth factor in the development of carcinomas. Characterization of the local expression of the gastrin receptor is ongoing.

HYDROLYSIS OF TYROSINE O-SULFATE IN ACIDIC SOLUTIONS
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Tyrosine O-sulfation is a common posttranslational modification of peptides and proteins transported through the Golgi apparatus, mediated by two enzymes designated Tyrosylprotein Sulfotransferase (TPST-1 and TPST-2). The biological function of tyrosine sulfation is modulation of protein-protein interactions as seen for the peptide hormone cholecystokinin (CCK) where tyrosine sulfation is necessary for binding to the CCK_A receptor. In addition sulfation regulates proteolytic processing, and secretion rates of secretory proteins. Tyrosine sulfation is not identified during Edmann sequencing, because the sulfate ester is very acid labile and rapidly hydrolyses to tyrosine in strong acidic solutions (pH < 1). Many protein purification and preparation procedures involve acidic solutions at slightly higher pH values, but little is known about the hydrolysis rate at these acid concentrations. In the present study sulfated gastrin-17, caerulein and drosulfokinin were used as models for tyrosine sulfated peptides. The sulfated peptides were incubated in acidic solutions with a pH range of 1 to 3, at different physical parameters i.e. temperatures, incubation times and with different acids. Sulfated and nonsulfated peptide fractions were separated and quantified by anion-exchange chromatography performed on a FPLC system. The results showed only marginal

hydrolysis of sulfated gastrin-17 in triflouroacetic acid (TFA) at pH 1 to 3 when incubating at room temperature or lower e.g. in 0.5% TFA (pH 1.3) the half-life of the reaction measured at 37ºC was 73.5 hours. No difference in hydrolysis rate was found between the three acids HCl, formic acid (FA) or TFA. The acid hydrolysis of the three sulfated peptides were compared, and it was shown that hydrolysis rate was dependent on the primary amino acid composition of the peptide. When identifying new targets of tyrosine sulfation, it is of major importance to avoid hydrolysis of the acid labile sulfate group and this study serves as a reference for handling sulfated peptides and proteins in aqueous acidic solutions.

NEUROENDOCRINE ORIGIN OF GASTRIC CARCINOMAS

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It has been known that hypo-/anacidity disposes to gastric cancer. Although not specified in most publications, there indications that these cancers occur mainly in the oxyntic area. In the seventies it was realized that carcinoids originating from the ECL cell developed in the oxyntic mucosa of patients with hypergastrinemia whether due to anacidity or gastrinoma. With the description of ECL carcinoids in the rats treated with the proton pump inhibitor (PPI) omeprazole, the interest in the ECL cell in gastric carcinogenesis increased dramatically. It was also shown that long-term treatment with histamine 2 (H_2) blockers increased such tumours not only in the rat, but also in mice. It was also clear that it was not so easy to differentiate between ordinary adenocarcinomas and ECL cell derived carcinomas, as exemplified by the gastric tumours in mastomys as well as in the rat. We started to study the role of the ECL cell in human gastric carcinomas in the late eighties, and in 1991 and 1998 we published that an important proportion of gastric carcinomas of diffuse type originates from the ECL cell. Later, by applying tyramide signal amplification in immunohistochemistry we could show that a larger proportion of these tumours were of ECL cell origin and thus gastrin dependent. Moreover, most of the gastric carcinomas in patients with atrophic gastritis/pernicious anaemia actually develop from the ECL cell. We were also able to show that a typical carcinoid in a patient with pernicious anaemia with time developed into a highly malignant neuroendocrine carcinoma, and very recently we could report that the signet ring cells in gastric carcinomas actually are neuroendocrine derived. In parallel we have developed the Japanese cotton rat model to show the importance of gastrin in gastric carcinogenesis.

In conclusion, the ECL cell and thus gastrin are important in gastric carcinogenesis.

GASTRINOMAS AND CCKOMAS: RECENT ADVANCES

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Gastrin-immunoreactivity/expression has been described in a number of different tumors, however relatively few are gastrin-secreting (gastrinomas) causing the Zollinger-Ellison syndrome (ZES). In the last few years there have been numerous advances in the understanding of gastrinomas. Recent studies provide insight into their development in MEN1 and sporadic cases and describe them in extra-abdominal locations for the first time. Advances in their imaging particularly with the use of octreoscanning have allowed better definition of their extent and biologic behavior and long-term studies are providing important insights into their natural behavior. Medical treatments of the gastric acid hypersecretory state is now highly effective in almost every patient. Recent surgical studies have provided a number of important insights and treatment advances including the increased appreciation of duodenal gastrinomas, the occurrence of lymph node primary gastrinomas and the possibility of surgery extending life. Long-term studies of patients with advanced disease are providing insights into which patients should be treated and providing prognostic factors to determine the aggressiveness of treatment. Lastly, molecular studies as well as a number of gene array studies are providing important insights into identifying patient whose disease pursues an aggressive course.

CCK-immunoreactivity/expression has been described in a small number of tumors. In contrast to gastrinomas it is not established that these tumors cause a specific syndrome.

Each of these areas will be briefly reviewed.