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NON-NEURONAL MAMMALIAN TACHYKININ EXPRESSION

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1. ABSTRACT

Mammalian tachykinins are traditionally viewed as neuropeptides. This review describes the mammalian tachykinins and evidence for expression of these peptides by non-neuronal cells. Tachykinin expression is defined as evidence for gene transcription, peptide production, or peptide secretion. Since the functions of mammalian tachykinins have been amply reviewed, the biological roles of these peptides will be noted briefly, with emphasis on immune cell action. Of particular interest is the predicted existence and non-neuronal expression of new mammalian tachykinins - hemokinin 1, the endokinins and C14TKL-1. Synthetic forms of these peptides have high affinity for the NK_1 receptor, the protein traditionally associated with substance P binding. By acting on the same "substance P" receptor, these tachykinins have the potential for promoting similar post-receptor functions. The structure and action of representative non-mammalian tachykinins acting on mammals are also presented. These peptides, of interest in their own right, also appear to exhibit selectivity for the NK_1 receptor. They strengthen the notion that multiple ligands may be capable of binding to one receptor, NK_1, effecting similar cellular responses.

2. INTRODUCTION

The tachykinin family of peptides (1) typically share two characteristics: a C-terminal sequence of F-X-G-L-M and amidation of the C-terminal Met residue. The X in the C-terminal sequence is one of four residues: F, Y, V, or I. Tachykinins are relatively short peptides with the well known mammalian substance P (reviewed in 2-5) containing 11 amino acid residues. The tachykinins have a variety of pharmacological actions both in the central nervous system (reviewed in 2-5) and in the periphery (reviewed in 6-8).

Tachykinins are produced as preproproteins and are processed and secreted via the classic secretory pathway. A signal (pre) sequence directs the nascent polypeptide to the endoplasmic reticulum where preprotachykinins are converted to protachykinin. Protachykinins are significantly larger than the functional tachykinin. In humans, for example, proneurokinin B is a 121 residue polypeptide (9). Protachykinins are sorted in the Golgi and proteolytically converted and amidated in secretory vesicles to yield the bioactive peptide. Secretion may be controlled by external signals, with the peptides acting locally in an autocrine, or paracrine fashion. Following secretion, the peptide either binds to a specific tachykinin receptor, or is degraded by membrane-bound extracellular proteases such as endopeptidase-24.11 (neprilysin) (10). Receptor binding results in G-protein activation and the production of inositol triphosphate (IP_3) and diacylglycerol (DAG) (11). This could give rise to an increase in cytosolic Ca^{2+}, or other intracellular events such as the activation of specific transcription factors like NF-kappaB. Bound receptors are rapidly endocytosed, the ligand degraded and the receptor returned to the plasma membrane. The turnover rate for tachykinins can be anywhere from 3-4 hours to 4 or 5 days if axonal transport is required (12).

Until recently, the mammalian tachykinins consisted of substance P, neurokinin A (NKA) and neurokinin B (NKB) with differential splicing and posttranslational processing also producing the neurokinin A-like peptides, neuropeptide gamma and neuropeptide K.

Table 1. Tachykinins and their amphipathic structure

Tachykinin	Amino acid sequence indicating the amphipathic structure[1]
Substance P	R P K P Q Q FFGLM-NH_2
Hemokinin-1 (mouse)	R S R T R Q FYGLM-NH_2
Endokinins A & B (human)	T G K A S Q FFGLM-NH_2
C14TKL-1 (human)	R H R T P M FYGLM-NH_2
Neurokinin A	H K T D S FVGLM-NH_2
Neurokinin B	D M H D F FVGLM-NH_2
Sialokinin I (mosquito saliva)	N T G D K FYGLM-NH_2
Physalaemin (frog skin)	E A D P N K FYGLM-NH_2
Virokinin (bovine viral)	R N S T K K FYGLM-NH_2

[1] Basic amino acid residues are indicated as red letters. Acidic amino acid residues are indicated as blue letters. Uncharged, polar amino acid residues are indicated as green letters. Hydrophobic amino acid residues (including glycine and tyrosine) are indicated as black letters

Substance P, NKA and NKB have been termed neurokinins because of their abundance in the central nervous system. Additional tachykinins are now thought to be present in mammals, based on the presence and transcription of novel tachykinin genes, and the biological activity of the tachykinin. It is also noted that some nonmammalian organisms such as certain species of mosquito and frog, produce tachykinins that act on mammals. Table 1 shows the amino acid sequences of substance P, NKA and NKB, the new tachykinins hemokinin 1, endokinins A and B and C14TKL-1, and examples of tachykinins that act on mammals. The tachykinin sequences shown in Table 1 are presented using color coded residues to visualize their amphipathic structure. The N-terminal portion of the tachykinin is hydrophilic and is unique for each of the illustrated tachykinins. This portion of the tachykinin appears to be less involved in high affinity binding to its receptor. For example, the electron paramagnetic resonance spectrum of a receptor bound substance P analog indicates the N-terminal lys-3 portion is highly flexible (13), an indication of little involvement in binding. The C-terminal portion of the peptide is a highly conserved, amidated, hydrophobic 5-residue sequence required for receptor binding.

The three mammalian tachykinin receptors are termed NK_1 (neurokinin receptor 1), NK_2 and NK_3 (reviewed in 14-16). Tachykinin affinity for these receptors is strongly associated with the identity of the second residue of the C-terminal pentamer. The mammalian tachykinins can be divided into two groups based on the amino acid residue at this second position (1): In the first group are tachykinins with an aromatic phe, or tyr residue. This set includes substance P, the new tachykinins, hemokinin-1, endokinins A&B, and C14TKL-1, as well as the above selected nonmammalian tachykinins. These peptides are reported to have high affinity for NK_1, suggesting this is a central characteristic of tachykinins with phe or tyr at this second position. The second class of mammalian tachykinins, NKA and NKB contain the aliphatic val at the second position, and have higher affinity for NK_2 and NK_3, respectively. It should be noted that at elevated concentrations, tachykinins will bind to receptors of lower specificity (14-16), suggesting that some *in vivo* ligand-receptor interactions could be based on proximity.

3. MAMMALIAN NON-NEURONAL TACHYKININ EXPRESSION

The following sections examine the non-neuronal expression and secretion of mammalian tachykinins, and provide brief examples of biological activity, or proposed biological function, with emphasis on inflammation and immunomodulation.

3.1. Substance P

Substance P is encoded by the tachykinin 1 (Tac1) gene. Transcription of Tac1 produces preprotachykinin-A (PPT-A) RNA (16), which is converted into one of four PPT-A mRNA splice variants, alpha, beta, gamma or delta (17). All four of the mRNA variants code for a protachykinin polypeptide containing substance P, with the beta and gamma transcripts also encoding neurokinin A. As described earlier, substance P is secreted and is thought to either bind to its preferred receptor, NK_1, or is degraded by extracellular proteases. The expression and secretion of substance P has generally been attributed to neurons, defining substance P as a neurotransmitter of the central and peripheral nervous systems. In the 1980s, however, substance P was suggested to have proinflammatory properties (18), and this new putative function prompted a search for the peptide and its mRNA in immune cells. Substance P would then be directly involved in immune cell communication and modulation.

Aliakbari *et al.* (19) chromatographically identified substance P in purified human eosinophils, and estimated the concentration at 21 fmol per 10^7 cells. Weinstock *et al.* (20) also demonstrated the production of substance P in this cell type. Mice infected with schistosomiasis often produce granulomas in the liver, and using *in situ* oligonucleotide hybridization, PPT-A mRNA was localized to the granuloma eosinophils. Substance P could be extracted from the granulomas and identified by its immunoreactivity and elution profile on HPLC. Eosinophil release of substance P could be promoted by the calcium ionophore A23187, or histamine (21). It was concluded that substance P was present in granulomas as a result of preprotachykinin expression in eosinophils, that substance P secretion could be stimulated by both pharmacological and physiological mediators, and that substance P may stimulate particular aspects of the immune system.

Pascual and Bost (22) demonstrated that the murine macrophage cell line P388D1 secreted a peptide with characteristics indistinguishable from substance P. An anti-substance P monoclonal antibody was used to affinity purify the secreted substance P and the peptide shown to be chemically similar to substance P by gel filtration and by reverse-phase HPLC. Synthetic substance P enhances secretion of an IL-1-like activity from P388D1

macrophages. When these macrophages were cultured in the presence of anti-substance P antibody, IL-1-like cytokine production was decreased, suggesting macrophage function was modulated by substance P in an autocrine, or paracrine fashion. The presence of substance P and its involvement in autocrine, or paracrine regulation was supported in a subsequent study using isolated rat macrophages (23). LPS upregulated PPT-A mRNA expression in rat macrophages, and secretion of substance P could be detected using anti-substance P monoclonal antibody. Additionally, PPT-A mRNA expression was promoted in adherent mononuclear leukocytes (possibly macrophages) isolated from mouse mesenteric lymph nodes following oral intubation with *Salmonella dublin* (24). These results support the hypothesis that substance P is a proinflammatory modulator of the immune response acting locally in an autocrine or paracrine fashion, and indicate that stimulation, such as the addition of LPS to the medium, may be required for expression.

Castagliuolo *et al*. (25) injected rat ileal loops with *Clostridium difficile* toxin A and after 1 hour isolated lamina propria macrophages (LPMs). After 2 hours in culture, LPMs from toxin A-injected loops, but not control loops showed increased levels of alpha, beta and gamma substance P mRNA and after 6 hours, substance P could be detected in the culture medium (~100 fmol/10^7 LPMs). The authors suggest that the released substance P would likely bind to appropriate cell receptors locally *in vivo*, perpetuating the inflammatory response to toxin A-induced enteritis.

Using RT-PCR, PPT-A mRNA was detected in isolated macrophages (26-29), dendritic cells (30), lymphocytes (31, 32) and fetal microglia (29, 33). As might be anticipated, substance P was identified in these immune cells by enzyme-linked immunoassay (26, 30, 31, 33), and in some instances selected stimuli produced low levels of secretion of substance P (i.e. fmols/10^6-10^7 cells). For example, capsaicin (26) and morphine (34) stimulated substance P secretion from human monocytes and macrophages. Capsaicin also promoted the release of substance P from PHA-stimulated human peripheral blood-isolated lymphocytes (31). These studies demonstrate that PPT-A mRNA and low levels of substance P can be produced by non-neuronal cells with the potential of locally modulating immune cell function. It has been suggested that leukocyte production of substance P following stimulation could be the result of immune stimuli that differ from those required for neuronal peptide production, or could reflect the need to enhance the immune response in tissues with limited innervation by peptidergic neurons (7). Thus substance P would contribute to the development of an immune response in the absence of neuronally-derived peptide.

Enhancement of the pro-inflammatory response by substance P (for reviews see 2, 6, 7, 35-37) would vary, depending on the particular immunogen, pathogen (38), or stress-related change (39). This requires the presence of the "substance P" receptor, NK_1, on a variety of leukocyte types, confirmed by the presence of NK_1 mRNA and functional protein in these cells (summarized in ref. 2). NK_1 mRNA was detected in human monocytes (34), macrophages (26), lymphocytes (31, 32, 34) and fetal microglia (33), and using anti-NK_1 antibodies, the receptor protein was detected on professional antigen presenting cells including macrophages (40), dendritic cells (41) and microglia (42). At low nanomolar concentrations, the binding of substance P promotes a variety of pro-inflammatory responses. In macrophages, an increased respiratory burst producing reactive oxygen intermediates is induced (43, 44). The addition of substance P to human blood monocytes releases the proinflammatory cytokines IL-1, IL-6 and TNF-alpha (45). LPS-stimulated secretion of IL-6, TNF-alpha and IL-1beta is boosted by preincubation of mouse peritoneal macrophages with substance P (46). Similarly, the peptide synergizes with LPS to augment IL-12p70 secretion (47). In parallel to its pro-inflammatory action, substance P diminishes LPS and IFN-gamma stimulated production of the immunosuppressive cytokine, TGF-beta-1 (48) and down-regulates macrophage production of anti-inflammatory somatostatin (49) These results support the view that substance P acts through NK_1 to stimulate, or augment the pro-inflammatory immune response.

There is evidence that substance P may act as a lymphocyte differentiation or proliferation factor. Early studies indicated that substance P could promote PHA-stimulated human T cell proliferation (50). Similarly, Stanisz *et al*. (51) showed that substance P enhanced the secretion of IgA from concanavalin A stimulated lymphocytes. Further studies showed that substance P, at subnanomolar concentrations, optimally enhanced LPS-stimulated IgM and IgA secretion from appropriate B lymphoma cell lines (52-54). In normal B cells purified from spleen or Peyer's patches, subnanomolar concentrations of substance P enhanced LPS stimulation of IgG3 and IgM secretion 5-10 fold (55). Similarly, when IL-6 was used as a stimulator, substance P enhanced the secretion of IgA and IgG (56). More recently, substance P was shown to reduce T cell adhesion to fibronectin (57) and induce atypical cytokine secretion from antigen-activated Th1 and Th2 cell lines, suggesting tachykinins can influence T helper cell phenotype (58). It is concluded that substance P is capable of co-modulating the phenotype and activity of a variety of types of lymphocytes.

3.2. Neurokinin A (NKA)

Like substance P, mRNA encoding neurokinin A is derived from PPT-A RNA transcribed from Tac1 (17). PPT-A RNA contains 7 exons, with exon 3 encoding substance P and exon 6 encoding neurokinin A (29, 33). Only the beta and gamma mRNA transcripts are differentially spliced to include exon 6 and therefore, as mentioned previously, only these two mRNAs encode NKA. N-terminal extensions of NKA termed neuropeptide K (59) and neuropeptide gamma (60) can be produced by the beta and gamma transcripts, respectively. Any non-neuronal cells producing these transcripts are capable of making, packaging and secreting substance P, NKA and neuropeptides K or gamma.

Beta and gamma PPT-A mRNA has been detected in human peripheral blood monocytes (26), monocyte derived macrophages (26) and isolated human peripheral blood lymphocytes, stimulated for 72 hr in culture with phytohemagglutinin and further treated with IL-2 (31). These transcripts were also detected in mouse intestinal lymphocytes (32) and human fetal microglia cells (33). Lambrecht *et al.* (30) demonstrated that bone marrow-derived dendritic cells produced the gamma PPT-A transcript. These results indicate that many of the same leukocytes thought to produce substance P are also capable of producing and secreting neurokinin A. The emphasis to date, however, has been on the detection of substance P and NK_1 from these cell types, with little attention to NKA and its high affinity receptor, NK_2.

3.3. Neurokinin B (NKB)

Tac2 produces the peptide neurokinin B in the mouse, whereas in humans (and rats), the gene producing NKB has been named Tac3. Human Tac3 has 7 exons and appears to produce a single PPT-B mRNA transcript encoding the 121 residue proneurokinin B which is processed to the biologically active 10 residue NKB (9). Neurokinin B has been considered a neuropeptide, secreted by central and peripheral neurons, acting on its high affinity receptor, NK_3.

Recently, NKB of non-neuronal origin has been reported in the placenta during pregnancy (for reviews, see 61, 62). RT-PCR was used to detect the presence of PPT-B during the first trimester of pregnancy, with the transcript increasing 5-fold near termination (9). *In situ* hybridization using labeled antisense riboprobes located the NKB mRNA to the outer placental syncytiotrophoblasts, a region, like the placenta as a whole, that is not innervated. These cells are positioned for secretion of NKB into the maternal circulation, and small but significant amounts of immunoreactive NKB could be detected in the maternal blood at early and late stages of pregnancy. The authors suggest that NKB would reduce blood flow to the liver, thereby increasing flow to the uterus and placenta. NKB could simultaneously act as a vasodilator in the fetal placental circulation (63). Plasma concentrations of NKB were greatly elevated in patients with pregnancy-induced hypertension and pre-eclampsia (9). The elevated levels of NKB, by constricting blood flow through the mesenteric and portal veins, would increase blood pressure and possibly be a direct cause of pre-eclampsia.

3.4. Hemokinin 1 (mouse HK-1)

The recently discovered mouse Tac4 gene contains four exons encoding PPT-C RNA (64). This RNA appears to be spliced in the mouse to yield a single mRNA species, producing a 128 residue preproprotein with the requisite processing information for the release of the substance P-like peptide hemokinin-1 (HK-1). Zhang *et al*. (64) used RT-PCR followed by Northern blot analysis to demonstrate expression in mouse bone marrow and thymus, also providing evidence that in bone marrow, HK-1 expression was largely restricted to the B cell lineage. RT-PCR expression profiling in the mouse has considerably expanded the repertoire of tissues containing HK-1 mRNA. Kurtz *et al*. (65) report moderate to strong signals from brain, spleen, stomach, skin and lactating breast, with weaker expression in other peripheral tissues. Using real-time quantitative PCR, Duffy *et al.* (66) detected HK-1 mRNA in the majority of peripheral tissues examined including spleen, ovary and uterus, with expression in the uterus confirmed by Pintado *et al.* (67). Examination of a variety of brain regions demonstrate the presence of HK-1 mRNA, but this expression is relatively weak compared to that of substance P mRNA (66). Cell and tissue production and expression of the HK-1 peptide has not as yet been reported due to the lack of specific anti-HK-1 antibodies.

Hemokinin-1 was synthesized and used in a variety of assays to analyze receptor binding and peptide activity. Like substance P, HK-1 has high affinity, and therefore specificity for NK_1. Morteau *et al.* (68), using the human NK_1 receptor, report a K_d for murine HK-1 of 0.11 nM and for substance P, a K_d of 0.16 nM. Within experimental error limits, these K_d's are the same. Similar binding affinities were reported by Kurtz *et al.* (65), Bellucci *et al*. (69) and Duffy *et al.* (66). As expected, substance P and hemokinin-1 at 100 nM concentrations increase cytosolic Ca^{2+} and cross-desensitize in NK_1 expressing cells (68). The EC_{50}s for Ca^{2+} mobilization and IP_1 accumulation are reported to be 0.6 and 0.47 nM, respectively, virtually identical to the measured substance P values (65). Similar EC_{50}s are reported by Duffy *et al.* (66). Despite the dissimilarity in amino-terminal sequences, these data strongly suggest that both HK-1 and substance P have the capacity to act on NK_1 *in vivo.*

HK-1 and substance P have similar pharmacological profiles. Concentration dependent contractions of NK_1-containing rabbit jugular vein were the same for HK-1 and substance P, and these contractions occurred at much lower concentrations than for rabbit pulmonary artery (NK_2), or rat portal vein (NK_3) (70). Bellucci *et al*. (69) report that in rat urinary bladder, HK-1 was similar, but about 3-fold less potent than substance P in producing contractions. *In vivo*, the potency of HK-1 was the same as substance P in generating dose-dependent salivary gland secretion and reduction in blood pressure. Duffy *et al*. (66) report that the minimum effective dose to produce foot tapping in gerbils was 10 and 3 nmoles for HK-1 and substance P, respectively, while for scratching in mice, the minimum effective dose was 1 nmole of each peptide. These data are supportive of the hypothesis that NK_1 is a high affinity receptor for HK-1 *in vivo.*

In addition to the potential substance P-like activities, HK-1 may be particularly important in B cell (64) and T-cell (71) development. In bone marrow, PPT-C mRNA expression was largely confined to B lineage cells (64). HK-1 promoted both the survival and proliferation of B cell precursors, the same cells producing HK-1, suggesting that the peptide is an autocrine factor playing a critical role in B cell development. Similarly, HK-1 influenced T-cell development at early stages of differentiation. In fetal thymus organ culture, HK-1 enhanced proliferation of T-cell precursors and increased the number of thymocytes. For both B-cell and T-cell precursors, NK_1 antagonists

reduced cell survival, which could be partially reversed by the addition of HK-1. It might be expected that all potentiating effects of HK-1 described for pre-B and pre-T cells would be reproduced by substance P. In some instances, however, the enhancing properties of HK-1 were uniquely attributed to this peptide. For example, HK-1 induced proliferation of IL-7 expanded B cell precursors, but substance P lacked this activity. These results would support the hypothesis that different tissues might have separate NK_1 subtypes, or different receptors for HK-1 and substance P.

3.5. Endokinins A and B (human EKA and EKB)

Based on the mouse sequence, the homologous Tac4 gene was located in the human genome and transcripts identified by analysis of cDNA (72). In humans, Tac4 expression is predicted to produce a PPT-C RNA that is differentially spliced to yield one of four mRNAs, alpha, beta, gamma, or delta. Relative to mouse, human PPT-C RNA contains a short, internal additional exon retained in the human alpha and gamma PPT-C mRNA transcripts.

It is not clear whether translation and processing of the resultant proproteins would yield an HK-1-like peptide, as found for mouse. The dibasic proprotein processing site at the amino-terminus of mouse HK-1 is absent in the human sequence. Kurtz *et al.* (65) propose that processing enzymes would make use of two monobasic cleavage sites to generate an 11-residue HK-1 peptide as in mouse, as well as an 8-residue truncated form (HK-1, residues 4-11). Page *et al*. (72) argue that the absence of the dibasic N-terminal processing site would shift the cleavage to a new site further toward the N-terminus of the proprotein, yielding peptides of 47 and 41 residues. These two peptides with the signature FFGLM tachykinin sequence at the C-terminus have been termed endokinins A and B (EKA and EKB), so named because of their peripheral production and endocrine/paracrine activity. Two other peptides, EKC and EKD would also be generated, but would lack the signature tachykinin sequence, and do not exhibit tachykinin-like activity. One striking observation is that regardless of the length of the human HK-1/EKA/EKB N-terminus, this portion of the human sequence differs substantially from that of mouse HK-1. There appears to be very little evolutionary pressure to maintain a specific N-terminal sequence.

Human Tac4 transcripts are present in a variety of tissues and cell lines. Kurtz et al. (65), using RT-PCR noted significant expression in the heart, skeletal muscle, skin and thyroid. Trace amounts of human PPT-C mRNA could be detected in the uteri of both pregnant and nonpregnant women (73). Alpha-PPT-C mRNA was detected in adrenal gland and fetal liver, beta-PPT-C mRNA in heart, liver, bone marrow, prostate, adrenal gland and testis, and delta and gamma PPT-C mRNA in adrenal gland and placenta (72). Detection of transcripts in a variety of human peripheral tissue cell lines support the hypothesis that these tachykinins are non-neuronally expressed.

Evidence for the production or secretion of human HK-1/EKA/EKB is limited. Page *et al.* (72) produced antibodies against the N- and C-termini of EKB and used these two antibodies in a sandwich ELISA to detect the presence of EKB in human term placental extracts. They found 7.52 fmol of EKB per gram of tissue.

"Human HK-1" (either synthetic TGKASQFFGLM-NH_2, or synthetic GKASQFFGLM-NH_2) generally acted like substance P or mouse HK-1 in binding and activity assays. Human HK-1 has high affinity for the "substance P" receptor, NK_1. Binding affinity for human NK_1 was slightly reduced compared to substance P or mouse HK-1, but was still substantially greater than for NK_2, or NK_3. In NK_1-transfected CHO cells, half-maximal concentrations for Ca^{2+} mobilization and IP_1 accumulation were virtually the same for substance P, mouse HK-1 and human HK-1 (65). Page *et al.* (72) report that in rats both substance P and human HK-1 reduce arterial blood pressure, promoting dose-dependent hindquarter vasodilation, mesenteric vasoconstriction and tachycardia. There were no significant dose-dependent differences between substance P and human HK-1. The authors conclude human HK-1/EKA/EKB may be responsible for actions in the periphery formerly attributed to substance P.

3.6. C14TKL-1

Jiang *et al.* (74) developed a homology search application, termed PepPat, that is designed to search for short peptide sequences within a database. The application, combining a pattern search with a similarity score against a query peptide, identifies possible matches from expressed regions of a genome. The authors used PepPat to search for tachykinin sequences. Specifically, a portion of the neurokinin A sequence including the C-terminal proteolytic cleavage site "FVGLMGKR" was used as the query sequence, and "XXXXMG[KR][KR] used as the pattern, to search a virtual translation of the Incyte Lifeseq EST database (Incyte Genomics, Palo Alto, CA). One high-scoring hit was "FYGLMGKR". Using BLAST, the expressed sequence mapped to human chromosome 14. The peptide sequence was translated N-terminally and based on a putative proteolytic cleavage site, the peptide was suggested to have the sequence"RHRTPMFYGLM-NH_2." This peptide was designated chromosome 14 tachykinin-like peptide 1 (C14TKL-1).

To establish the biological presence and relevance of this peptide, the authors assayed for mRNA expression and synthetic C14TKL-1 activity. One-step quantitative RT-PCR was used to demonstrate that the gene is expressed in many human organs and in numerous regions of the brain. The authors particularly noted that C14TKL-1 mRNA was present at elevated levels in peripheral tissue, and was abundant in liver. NK_1 transfected CHO cells showed a dose-dependent rise in cytosolic Ca^{2+} upon addition of synthetic C14TKL-1. This rise in internal Ca^{2+} was indistinguishable from the response to substance P, with half-maximal values of 1.0 and 0.8 nM, respectively. These values are similar to the K_d reported for substance P binding to the NK_1 receptor (0.1 - 1.0 nM). The authors conclude, based on mRNA expression and peptide activity, that C14TKL-1 is a likely addition to the list of *in vivo* NK_1 agonists.

4. TACHYKININS ACTING ON MAMMALS

The following section briefly describes representative non-mammalian tachykinins that are thought to act on mammals. These peptides are particularly intriguing since they contain tyr in the second position of the tachykinin C-terminal pentamer, a characteristic of two of the new tachykinins, mouse HK-1 and human C14TKL-1. In the context of tachykinin structure, these peptides provide evidence that tachykinins found in nature may differ substantially at their N-terminus while retaining high affinity for the NK_1 receptor.

4.1. Virokinin

Virokinin is a tachykinin produced from the fusion protein of bovine respiratory syncytial virus (BRSV) (75). By processing of a viral membrane protein, a peptide is released that is further proteolytically modified and amidated. The 20 residue virokinin is proposed to have the sequence GIPELIHYTRNSTKKFYGLM-NH_2. The peptide contains an N-linked glycosyl group that does not appear to affect biological activity. The authors suggest that glycosylation may help protect virokinin from the action of endopeptidase-24.11. The authors note that the sequence of virokinin is conserved in all BRSV strains, indicating that the virus benefits from the production of this peptide.

A monospecific antibody directed against the amidated C-terminus of virokinin was used on Western blots to demonstrate that native virokinin was secreted by infected Vero cells. Both the native peptide (in the form of conditioned medium) and synthetic virokinin were then tested in biological activity assays. GFP-tagged beta-arrestin 1 in NK_1 transfected HeLa cells was translocated to endosomes in the presence of 10 nM virokinin, or in the presence of conditioned, infected Vero cell medium. Similarly, GFP-tagged NK_1 transfected into CHO-K1 cells was internalized in the presence of 10 nM synthetic virokinin, or conditioned medium. These results demonstrate that virokinin binds with high affinity to NK_1, and, like substance P, activates NK_1 at low nanomolar concentrations. They also showed that at high concentrations (0.5 uM), synthetic virokinin induced strong, reversible smooth muscle contractions in isolated circular muscle from guinea pig stomach. These contractions were significantly reduced in the presence of an NK_3 inhibitor. Although binding to NK_3 may be of lower affinity, this result further supports the bioactivity of virokinin.

The authors suggest that virokinin mimics mammalian tachykinins, contributing to both the symptoms of BRSV infection and benefiting the virus by altering the host immune response. Substance P and NKA induce an influx of lymphocytes, edema formation, increased mucus secretion, histamine release and bronchoconstriction in the lungs during inflammation (76, 77). These symptoms would be magnified by the presence of virokinin. At the site of infection, the accumulation of high concentrations of virokinin could desensitize NK_1, down-regulating the immune response. This down-regulation could delay viral clearance.

4.2. Sialokinins I/II

Ribeiro (78) reported a vasodilatory activity in homogenates from the salivary glands of *Aedes aegypti*, the yellow fever mosquito. Reverse-phase HPLC suggested the activity resided in a peptide with approximately the same mass as substance P. A monoclonal antibody directed against the C-terminus of substance P appeared to cross react with the mosquito peptide in fixed salivary glands. Champagne and Ribeiro (79) purified this activity and found two peptides, sialokinin I, with the sequence NTGDKFYGLM-NH_2, and sialokinin II, with identical sequence except for the substitution of an Asp residue at position 1.

Sialokinins are thought to trigger a response by binding to NK_1. Sialokinins I & II were synthesized and their activity in a guinea pig ileum bioassay compared to the activity of substance P. Half-maximal contractile responses to the three peptides were virtually identical, with $K_{0.5}$ values of 6.58, 5.07 and 4.94 nM for sialokinin I, II and substance P, respectively. Although these concentrations are slightly greater than the K_d for substance P, guinea pig ileum may contain low affinity binding sites that would typically reduce the free ligand concentration, thereby modestly increasing the apparent half-maximal response values. Guinea pig ileum contains NK_1 receptors (80), thus the simplest conclusion is that the sialokinins bind to NK_1 with the same affinity as substance P, and, like substance P, induce a vasodilatory response.

There is little doubt that the most immediate and important action of the sialokinins is vasodilation. The mosquito benefits by increasing blood flow to the site of feeding. Immune responses to the sialokinins, however, could also be relevant to mosquito vector transmission. Champagne and Ribeiro (79) point out that the concentration of sialokinin required for smooth muscle contraction in the guinea pig ileum assay is the same as that needed for augmentation of macrophage responses by substance P. They suggest that promotion of macrophage activity at the site of mosquito feeding could result in macrophage infection, followed by macrophage dispersal of disease-causing parasitic vectors. Zeidner *et al.* (81) offer another potential role for sialokinins in viral transmission to the mammalian host. Mosquitoes were fed on flavivirus susceptible mice, or mice were injected directly with sialokinin. Four to seven days post-feeding or post-injection, stimulated lymphocytes were harvested and assayed for cytokine production. The Th1 cytokine IFN-gamma was down-regulated relative to control lymphocytes from congenic flavivirus resistant mice, whereas the Th2 cytokines IL-4 and IL-10 were up-regulated. The authors imply that sialokinin could locally promote a sub-optimal Th2 response to viral infection, increasing the likelihood of viral transmission.

4.3. Physalaemin

Numerous tachykinins, including physalaemin (82), have been isolated from frog skin (1). The venom glands on the backs of frogs and toads are the source of a variety of toxins, such as bufotoxin, whose uses include the

production of poison arrowheads. Although skin toxins confer an obvious advantage to an amphibian by reducing predation, exactly how physalaemin acts on mammals does not appear to be known (1).

Physalaemin contains the signature C-terminal tachykinin sequence FYGLM-NH_2 and is considered to be an NK_1 agonist. Regoli *et al.* (83) reported that the substance P receptor (NK_1) was particularly sensitive to substance P and physalaemin. More specifically, Vigna *et al.* (84) and Kitsukawa *et al.* (85) used canine chief cells to demonstrate that physalaemin was equal in potency to substance P in displacing radiolabeled substance P from its receptor. The IC_{50}s of 0.1-0.2 nM suggest the physalaemin has this same high affinity for NK_1 as substance P. Along with virokinin and the sialokinins, physalaemin is a further example of a naturally occurring tachykinin with high affinity for NK_1. These peptides support the view that NK_1 binding requires phe or tyr at position X of the signature FXGLM-NH_2 tachykinin C-terminal sequence, and indicates considerable sequence variability is permissible in the hydrophilic N-terminus.

5. PERSPECTIVE

There is compelling evidence that mammalian tachykinins are expressed in non-neuronal cells *in vivo.* Substance P is expressed in immune cells, neurokinin B in the placenta, and hemokinin 1 in B cells. The endokinins A and B, and C14TKL-1 are likely to be of non-neuronal origin since they are expressed in a wide variety of tissues, and in the case of the endokinins, are expressed in numerous peripheral tissue cell lines. It remains critical to prove the existence of the new tachykinins. For example, mouse HK-1 and human C14TKL-1 must either be purified from tissues and sequenced, or must be identified by development and use of monospecific antibodies. It will be of particular interest to define the relationship between the new mammalian tachykinins and their receptor(s). Synthetic forms of these peptides have high affinity for NK_1, the "substance P" receptor and at least two simple scenarios are possible for receptor-ligand interaction.

One possibility is obvious: the new mammalian tachykinins grouped with substance P may simply have high affinity for NK_1; that is, there is a single receptor capable of binding multiple ligands with high affinity. This is supported by the high affinity for NK_1 demonstrated by the three selected, naturally occurring nonmammalian tachykinins. This hypothesis is consistent with the C-terminal F (F/Y) GLM-NH_2 tachykinin sequence dictating high affinity NK_1 binding. What is the purpose of the N-terminal sequence? The N-termini have in common their hydrophilic nature. Since the amidated C-terminus is highly hydrophobic and presumably insoluble in aqueous solution, one might speculate that the N-terminus is merely present to provide the requisite peptide solubility. If this is the case then the new mammalian tachykinins would promote the same post-receptor functions as substance P.

Alternatively, ligand-receptor affinities may be locally matched, with each mammalian tachykinin binding to a preferred receptor state, or isoform. NK_1 affinity could be altered by coupling to different G proteins (86-88), or by the existence of different states of the receptor (i.e., phosphorylation states) (89). There is also the prospect for the discovery of new NK_1 isoforms as genomic sequences are analyzed. By matching tachykinin with receptor, there would be increased specificity for post-receptor functions and a greater prospect for the discovery of new roles for the mammalian tachykinins.

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