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Review

Hemokinins and endokinins

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Abstract. The mammalian tachykinins are a family of peptides that, until recently, has included substance P (SP), neurokinin A and neurokinin B. Since, the discovery of a third preprotachykinin gene (*TAC*4), the number of tachykinins has more than doubled to reveal several species-divergent peptides. This group includes hemokinin-1 (HK-1) in mouse and rat, endokinin-1 (EK-1) in rabbit, and EKA, EKB, human HK-1 (hHK-1) and hHK(4–11) in humans. Each exhibits a remarkable selectivity and potency for the tachykinin NK_1 receptor similar to SP. Their peripheral expression has led to the proposal that they are the endogenous peripheral SP-like endocrine/paracrine agonists where SP is not expressed. Moreover, their strong cross-reactivity with a specific SP antibody leads us to question many of the proposed locations and roles of SP in the periphery. Additionally, three orphan tachykinin gene-related peptides are identified on *TAC*4, in rabbit, EK-2 and in humans, EKC and EKD.

Key words. Endokinin; hemokinin; tachykinin; neurokinin; peptide; *TAC*4.

Introduction

The mammalian tachykinins are a family of peptides traditionally classified as neurotransmitters. They have included, until recently, the three members substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), all being short peptides of 10–11 amino acids in length. By definition these peptides all share the same conserved hydrophobic C-terminal region, FXGLM-NH_2, where X is always a hydrophobic residue that is either an aromatic or a β-branched aliphatic. The C-terminal region is central to the activation of each of the three known mammalian tachykinin receptors, NK_1, NK_2 and NK_3 [1, 2]. In contrast, at their N-terminals they possess divergent and hydrophilic regions believed to convey receptor specificity [3], whereby SP shows the highest affinity for NK_1, NKA for NK_2 and NKB for NK_3. The activation of the tachykinin receptors has been implicated in a wide variety of biological actions from smooth muscle contraction, vasodilatation, pain transmission, neurogenic inflammation, activation of the immune system and stimulation of endocrine gland secretion [4]. Furthermore, the conceived dogma that these tachykinins are exclusively restricted to nervous tissue exerting their effects, in the periphery, only by release from nerve endings has been challenged. They have been detected not only in non-neuronal cells such as immune [5] and inflammatory cells [6], but even in the placenta, a tissue totally devoid of nerves [7]. Especially, in the latter regard since there are no neuronal connections, its main action here is proposed to be endocrine rather than neuronal [7].

Historical perspective

The mammalian tachykinins can be traced back 73 years to 1931 with the discovery of SP by von Euler and Gaddum [8]. A ‘preparation’ (and hence the ‘P’ of SP) was purified from horse brain and intestine that was found to cause vasodilatation and stimulation of gut mobility in rabbits. Numerous attempts to isolate the pure form of SP over the next 40 years were unsuccessful. Nonetheless, in

1967, a peptide named sialogen that stimulated salivation was discovered in the bovine hypothalamus [9], and in the following year Lembeck and Starke [10] suggested this peptide may be the same as SP. In 1970, upon purification and comparison of sialogen's biological properties with those of impure preparations of SP, it was realized that these two peptides were indeed the same [11]. This enabled the chemical structure of SP to be determined as that of an amidated undecapeptide (table 1) [12], allowing its chemical synthesis [13] and enabling the production of a specific radioimmunoassay [14]. Over the next 10 years, SP was believed to be the only mammalian tachykinin even though there were some indications that this might not be entirely the case. Inference was drawn from the detection of immunoreactive tachykinin-like activity in mammalian tissues other than SP [15]. Additionally, eledoisin [16] and physalaemin [17], two tachykinins isolated in the early sixties from the Mediterranean octopus and the South American frog, respectively, were found to produce greater pharmacological responses on mammalian tissues than SP [18, 19]. In 1983, these suppositions gave way to confirmation of the existence of two further members of the mammalian tachykinin family both isolated from the porcine spinal cord. These were designated NKA [20–22] and NKB [20, 23]. There are also two biologically active N-terminally extended forms of NKA, neuropeptide K (NPK) [24] and neuropeptide gamma (NPγ) [25] (table 1). All of these tachykinins are encoded by two genes, preprotachykinin 1 (*TAC*1), encoding the precursors for SP, NKA, NPK and NPγ, and *TAC*3, encoding the precursor for NKB [26, 27]. The alternative splicing of *TAC*1 produces four messenger RNA (mRNA) transcripts yielding four distinct precursors (α, β, γ and δ) with SP encoded by all four, NKA encoded by β and γ*TAC*1, NPK by β*TAC*1 and NPγ by γ*TAC*1 [21, 28, 29] (table 2).

It could have been concluded that each of the three major mammalian tachykinins with its own preferred receptor had been found. Indeed, a further 17 years passed without the elucidation of any further tachykinins, nor were any further tachykinin receptors verified. Notwithstanding this, Severini et al. [30] commented that the number of mammalian species and tissues that tachykinin peptides had been isolated from was rather scant. In the lower vertebrates such as the fish five different tachykinins had been reported in the brain and six in the gut; in contrast, in amphibians nine different tachykinins had been observed [30]. However, in 2000, this picture was about to change with the discovery of a fourth mammalian tachykinin in the mouse that was named hemokinin-1 (HK-1) [31]. This breakthrough has enabled the realization of a completely new and diverse group of novel

Table 1. Amino acid sequences of the different groups of mammalian tachykinins, the neurokinins, endokinins and tachykinin gene-related peptides.

Tachykinin	Peptide sequence	Reference
Neurokinins		
SP	RPKPQQFFGLM-NH_2	[12]
NKA	HKTDSFVGLM-NH_2	[20, 21, 22]
NPK	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	[20, 23]
NPγ	DAGHGQISHKRHKTDSFVGLM-NH_2	[24]
NKA (3–10)	TDSFVGLM-NH_2	[38]
NKB	DMHDFFVGLM-NH_2	[25]
Endokinins		
Human		
EKA	DGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2	[35]
EKB	DGGEEQTLSTEAETWEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2	[35]
EKA/B	GKASQFFGLM-NH_2	[35]
hHK-1	TGKASQFFGLM-NH_2	[34]
hHK-1 (4–11)	ASQFFGLM-NH_2	[34]
Mouse/rat		
HK-1	(R)*SRTRQFYGLM-NH_2	[31*, 34*, 35]
Rabbit		
EK-1	GKASQFFGLM-NH_2	[N. M. Page, unpublished observations]
Tachykinin gene-related peptides		
Human		
EKC	KKAYQLEHTFQGLL-NH_2	[35]
EKD	VGAYQLEHTFQGLL-NH_2	[35]
Rabbit		
EK-2	VRGYQMGQRGLL-NH_2	[N. M. Page, unpublished observations]

tachykinins and, more unexpectedly, tachykinin gene-related peptides in the mammals. These developments form the keystone of this review.

Discovery of a third mammalian tachykinin gene (*TAC*4)

Mouse and rat *TAC*4

The discovery of murine *TAC*4 arose from a mRNA differential display screen to isolate new growth and differentiation factors involved in mouse B cell development [31], whereby Zhang et al. [31] predicted a 128-amino acid precursor protein primarily expressed in murine hematopoietic cells. This precursor contained the decapeptide sequence KRSRTRQFYGLMGKR with the tachykinin signature motif (FXGLM-NH_2) flanked by potential dibasic cleavage sites and an adjacent glycine at the C-terminus for amidation. This pattern is distinct to all tachykinin peptides, whereby the mature peptide is flanked initially both upstream and downstream by paired dibasic residues in its precursor protein that act as proteolytic cleavage sites for prohormone convertases [32]. Typically, following prohormone convertase action, a carboxypeptidase removes the C-terminal dibasic residues, and a peptidylglycine α-amidating enzyme converts the exposed glycine into a C-terminal amide [33]. The resulting mature HK-1 peptide was found to resemble most closely that of SP (table 1).

The rat *TAC*4 homologue was subsequently identified encoding a 170-amino acid precursor protein that displayed a 76% identity to that of the mouse [34]. More crucial was the fact that the rat TAC4 precursor also encoded a HK-1 peptide identical to that of the mouse [34]. Zhang et al. [31] and Kurtz et al. [34] predict this peptide to be RSRTRQFYGLM-NH_2. Page et al. [35] have predicted instead SRTRQFYGLM-NH_2, which does not contain an extra arginine at its N-terminus. Unlike SP, where an arginine residue is only left at the N-terminus because of the resistance of arginine-proline bonds to prohormone-con-

Table 2. Molecular biological data for the mammalian tachykinins.

Species	Gene	Chromsome position	mRNA transcript	cDNA transcript length (bps)	Precursor protein length (amino acids)	Peptides predicted on precursor	Accession number	Reference
Human	*TAC*1	7q21-q22	α*TAC*1	1048	111	SP	NM_013996	[21]
			β*TAC*1	1102	129	SP, NKA, NPγ	NM_003182	[21]
			γ*TAC*1	1057	114	SP, NKA, NPK	NM_013997	[28]
			δ*TAC*1	1003	96	SP	NM_013998	[29]
Human	*TAC*3	12q13-q21	α*TAC*3	902	135	NKB	AF537115	N. J. Bell and N. M. Page, unpublished observations)
			β*TAC*3	785	121	NKB	AF537118	[26, 27]
			γ*TAC*3	731	103	*	AF537121	N. J. Bell and N. M. Page, unpublished observations)
Human	*TAC*4	17q21.33	*TAC*4	225	68	hHK-1, hHK-1 (4–11)	AF521560	[34]
			α*TAC*4 v1	675	113	EKA, EKC	AF515828	[35]
			α*TAC*4 v2	657	107	EKB, EKC	AY471574	(N. M. Page, unpublished observations)
			β*TAC*4	624	96	EKB, EKD	AF515829	[35]
			γ*TAC*4	597	87	EKB	AF515830	[35]
			δ*TAC*4	564	76	EKB	AF515831	[35]
Mouse	*TAC*4	11 D cM	*TAC*4	1248	128	HK-1	AF515827, AF235035	[31, 35]
Rat	*TAC*4	10q31 cM	*TAC*4	1083	170	HK-1	AY471575, AF521561	[34]
Rabbit	*TAC*4	^	α*TAC*4	642	100	EK-1, EK-2	AY471576	(N. M. Page, unpublished observations)
			β*TAC*4	588	82	EK-1	AY471577	(N. M. Page, unpublished observations)

^ Position unknown, * Not applicable.

verting enzymes [36], this peptide does not contain this proline. Therefore, normal processing is expected to remove this arginine, making HK-1 a decapeptide starting with the first adjacent serine.

The mouse TAC4 precursor is encoded on four exons, while the rat precursor is encoded on five (fig. 1) [35]. In the rat, this additional exon produces the insertion of an undecapeptide sequence into the precursor protein (VHPIQSAERTG) downstream of the predicted HK-1 peptide. Additionally, the rat precursor protein is extended at its C-terminus by 31 amino acids. This extension is accounted for by the existence of an adenine nucleotide insertion into the coding region of rat exon 5 that effectively produces a frameshift mutation [N. M. Page, unpublished observations]. There has been no evidence of alternatively spliced variants in the mouse or rat alluding to the presence of additional tachykinins [31, 34, 35, 37]. However, recently, a splice variant in the rat placenta and adrenal gland missing exon 2 (representing ~10% of the mRNA population) was found [N. M. Page, unpublished observations]. Exon 2 encodes HK-1, where such a mechanism may serve as a posttranscriptional event to control the amount of peptide translated. A similar phenomenon in the human *γTAC*3 transcript occurs by the alternative splicing of exon 5 encoding NKB (table 2).

Human *TAC*4

The characterization of the mouse and rat T*AC*4 was followed by the isolation of a corresponding human *TAC*4 homologue. A bioinformatics approach identified two human genomic sequences from chromosome 17 containing two putative exons predicted to encode a 68-amino acid precursor protein [34]. A HK-1-like decapeptide (GKASQFFGLM) with the tachykinin signature motif was predicted to be encoded on this precursor [34, 35]. At the C-terminus, a dibasic cleavage site adjacent to a glycine has the potential to liberate this as an amidated peptide. Surprisingly, this peptide does not share complete homology with mouse or rat HK-1. In particular, there is a single amino acid substitution adjacent to the N-terminus of the HK-1-like peptide that changes arginine to threonine, destroying the N-terminal dibasic processing site. The N-terminal of this precursor also lacks additional dibasic residues, while in the tachykinin signature motif (FXGLM-NH_2), a substitu-

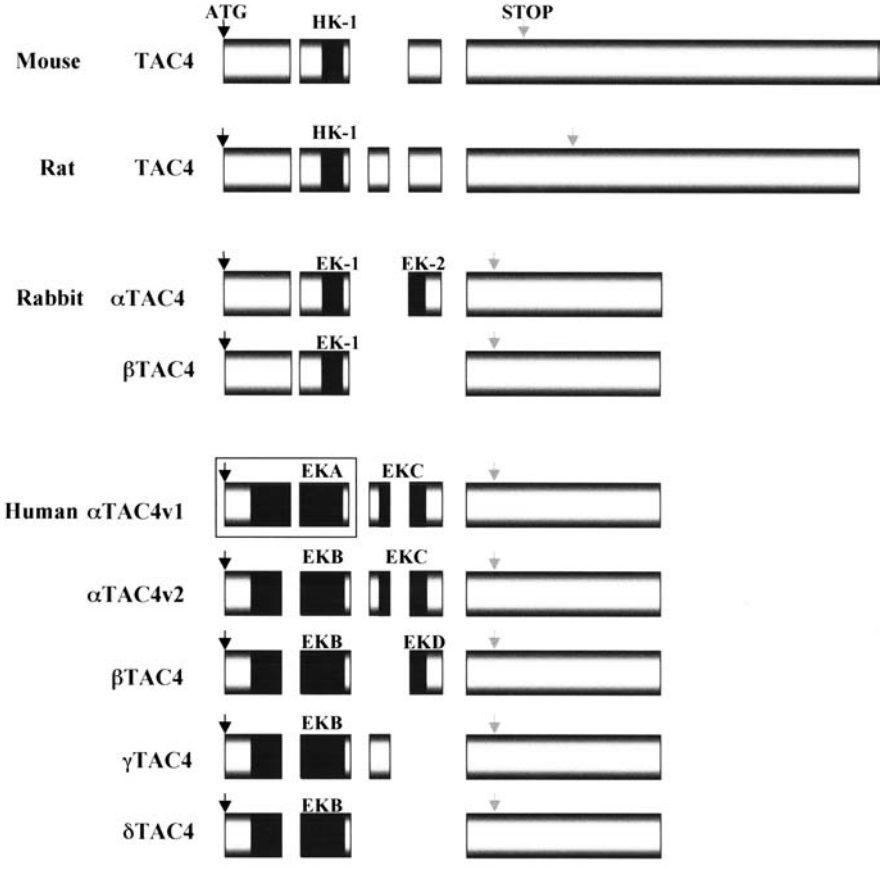

Figure 1. Exon organization of the mouse, rat, rabbit and human *TAC*4 genes showing the positions of the predicted peptides. □ Human exon organization of hHK-1 as predicted by [34].

tion in the X position from tyrosine in HK-1 to phenylalanine in humans produces a peptide with the last six residues identical to SP [34, 35]. These features are unusual in the mammalian tachykinins because all known tachykinins to date are identical across the species and flanked by dibasic residues. Two forms of the human HK-1-like peptide have been proposed by Kurtz et al. [34]. Each is based on monobasic cleavage at the N-terminal, resulting in hHK-1 (TGKASQFFGLM-NH_2) and hHK-1(4–11).

Nevertheless, the human *TAC*4 proposed by Kurtz et al. [34] is encoded only on two exons equivalent to the first two coding exons of the mouse and rat (fig. 1). Indeed, 3′ rapid amplification of cDNA ends (RACE) performed by Kurtz et al. [34] from human hypothalamus and thymus complementary DNA (cDNA) failed to identify a single clone that could be extended in the 3′ direction. On the contrary, Page et al. [35] identified a cDNA from human spleen that rendered a much longer 113-amino acid precursor protein predicted to be encoded on five exons. This included in humans three additional downstream exons with low identity to those of the mouse and rat. Furthermore, by the utilization of different combinations (inclusion or exclusion) of the third and fourth exons, this human *TAC*4 is spliced into four alternatively splice variants – α, β, γ and δ [35] (fig. 1). On these transcripts four different peptides are predicted, classified as endokinin A (EKA), EKB, EKC and EKD (tables 1, 2). EKB is encoded by all four transcripts (α, β, γ and δ*TAC*4), EKC by α*TAC*4 and EKD by β*TAC*4, whereas EKA has been found encoded so far only on α*TAC*4 [35]. EKA consists of 47 amino acids, while EKB is a 41-amino acid truncated form of EKA having a deletion of six amino acids. EKA and EKB are N-terminal elongated forms of hHK-1 (TGKASQFFGLM-NH_2) and hHK-1(4–11) [34].

C-terminal to EKA and EKB on the human TAC4 precursor are EKC and EKD, both tachykinin-like peptides (fig. 1). EKC consists of 14 amino acids, whereas EKD is a 14-amino acid N-terminally modified version of EKC (tables 1, 2). Both peptides are flanked by dibasic residues in the precursor protein and contain a C-terminal glycine, which converts to an amide. Whilst EKA and EKB both share the common C-terminal tachykinin signature motif, EKC and EKD possess a different C-terminal sequence. Exceptional to their tachykinin signature motif is the introduction in the X position of a hydrophilic glutamine amino acid, while a more conservative substitution is found in this motif, causing a transition from the C-terminal methionine to a leucine (i.e. FXGLM-NH_2 to FQGLL-NH_2) [35]. It is the alternative splicing of exons 3 and 4 that produces EKC and EKD, and, in essence, enables either EKA or EKB to be synthesized alone or in conjunction with either EKC or EKD [35]. This is a situation analogous to the alternative splicing of exons 4 and 6 of *TAC*1 producing either SP alone or along with NKA [21, 28, 29]. Nonetheless, corresponding second tachykinin-like peptides are not predicted in the mouse or rat. Intriguingly, there appears to be an evolutionary footprint, possibly demonstrating the origin from which EKC and EKD may have evolved (fig. 2A). Thus, the lack of these peptides in the mouse and rat may explain the reason why no splice variants in this region have been found in these species. In evolutionary terms, phylogenetic tree analysis of the mouse and human *TAC*1, *TAC*3 and α*TAC*4 precursor proteins shows *TAC*4 to be more closely related to *TAC*1 that encodes SP and NKA, than *TAC*3 encoding NKB (fig. 2B) [N. M. Page, unpublished observations].

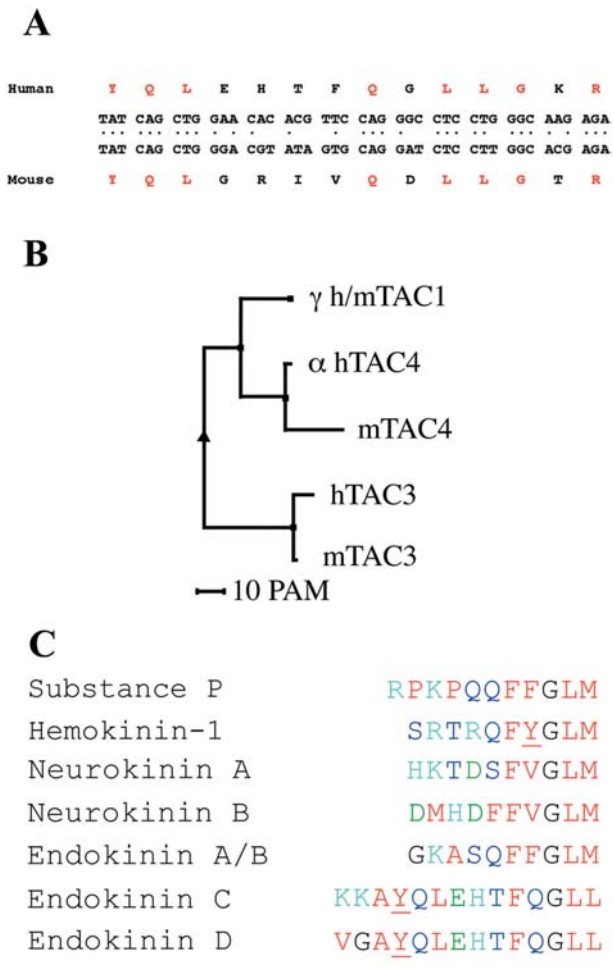

Figure 2. (*A*) Alignment of the nucleotide and amino acid sequences of the TAC4 precursors of the mouse and human in the region of the tachykinin gene-related peptides. (*B*) Phylogenetic tree of the mouse and human *TAC*1, *TAC*3 and *TAC*4 transcripts. The branch lengths indicate the evolutionary distances between the different genes. (*C*) Alignment of the amino acid sequences of the mammalian tachykinins, highlighting their different structures and properties. X, hydrophilic, neutral, polar. X = hydrophilic, negatively charged. X = hydrophilic, positively charged. X = hydrophobic, neutral, polar. X = neutral, aliphatic.

Rabbit *TAC*4

One question remaining unanswered was whether such tachykinin-like peptides (as EKC and EKD) are unique to humans. At which stage did these peptides first appear? Cloning of rabbit *TAC*4 cDNA from pooled lung, spleen and thymus revealed such an answer. Very interestingly, this has revealed a gene structure almost identical to that of human, alternatively spliced, to yield two transcripts α and β*TAC*4 (fig. 1, table 2). Both transcripts encode a peptide identical to that of the C-terminal decapeptide of EKA and EKB. Intriguingly, this peptide does possess a N-terminal dibasic cleavage site (KRTGKASQFFGLMGKR) with the potential to release the peptide TGKASQFFGLM-NH_2 without argument to how N-terminal cleavage may occur instead. This raises a further enticing question, the reason for the loss of the N-terminal dibasic cleavage site in humans. Moreover, the β*TAC*4 transcript was found to encode a 12-amino acid tachykinin-like peptide, VRGYQMGQRGLL-NH_2. The two peptides encoded on rabbit *TAC*4 have been termed endokinin 1 (EK-1) and EK-2, respectively [N. M. Page, unpublished observations] (table 1). The C-terminal signature motif of EK-2 is found to be even more disparate from that of the unclassical tachykinin signature found in EKC and EKD [35]. Consequently, these peptides appear unrelated to the tachykinins, with the common C-terminal motif being GLL-NH_2. Conceivably, EKC, EKD and EK-2 are more aptly called tachykinin gene-related peptides [39].

Peripheral mRNA distribution

Zhang et al. [31] first classified the tachykinin encoded on murine *TAC*4 as HK-1 because of its restricted expression in hematopoietic cells. Largely isolated to B lineage cells, its cellular expression was stage specific, with pro- and pre-B cell lines expressing *TAC*4, whereas those correlating to later developmental stages did not. No expression was found in the mouse brain, lung, heart, liver, spleen, thymus or kidney by Northern blot analysis, but polymerase chain reaction (PCR) did reveal expression in the thymus and more predominantly in the bone marrow. From their data, Zhang et al. [31] revealed an expression pattern for murine *TAC*4 primarily in (peripheral) hematopoietic cells rather than the predominant neuronal expression of the other known mammalian tachykinins. To date, these observations still stand but are now viewed within a much broader context.

More recently, semiquantitative PCR has demonstrated extensive *TAC*4 expression in a number of other murine peripheral tissues, including brain, spleen, stomach, lung, skin, breast, bone marrow, thymus, prostate, uterus, skeletal muscle, lymph node and eye [34, 35, 37]. Moreover, there are distinctions in the expression of murine *TAC*1 and *TAC*4, with *TAC*1 expression prolific in brain, heart, stomach and smooth muscle, whereas *TAC*4 is prolific in bone marrow, uterus and skeletal muscle [35]. These data have been corroborated with more sensitive real-time quantitative PCR, and in parallel with analysis of numerous regions of the brain, they reveal similar regional distributions for brain *TAC*1 and *TAC*4, though *TAC*4 was present at consistently lower levels [37]. Therefore, although HK-1 is not exclusive to the periphery, it is more abundantly expressed there, particularly in the absence of SP. This is illustrated in the rat by real-time quantitative PCR demonstrating the broad and distinct expression of *TAC*4 in the peripheral organs of the spleen, kidney and lung and also a number of endocrine tissues, including the testis, adrenal gland and placenta [N. M. Page, unpublished observations] (fig. 3B). On the contrary, *TAC*1 is expressed at extremely high levels in the brain. The expression of *TAC*4 in spleen and lung may represent expression in immune cells such as macrophages.

In humans, there is also wide expression of *TAC*4, with the first report detecting strong expression in the heart, skeletal muscle, skin and thyroid, with weaker expression in many other peripheral tissues [34]. Notably, this study used primers designed to span exons 1 and 2 (fig. 1). Yet, in humans, this does not take into account the extra level of complexity introduced by the different splice variants. Page et al. [35] have dissected this expression by designing specific PCR primers to overlap each unique exon junction. This showed α and β*TAC*4 to have very restricted expression patterns, α*TAC*4 being found only in the adrenal gland, fetal liver and very weakly in the spleen, and β*TAC*4 found in the heart, liver, bone marrow, prostate, adrenal gland and testis only. In contrast, γ and δ*TAC*4 were ubiquitously expressed in a range of human tissues, with the most prolific expression in the adrenal gland and placenta. Intriguingly, the adrenal gland was the only tissue found to express all four *TAC*4 transcripts and hence expected to produce all four endokinins. There was low expression of *TAC*4 in brain; conversely, high levels were found in the human placenta, a tissue completely devoid of neuronal input. Consequently, Page et al. [35] derived the name endokinins (EKA to EKD) for this group of peptides in line with their proposed peripheral endocrine roles rather than any neuronal role (cf. neurokinin). Reflective of this is the expression of γ and δ*TAC*4 in a wide variety of human peripheral cell lines; in contrast, *TAC*1 expression was limited to the adrenal cortical cell line, H295, and the acute myeloid leukaemia cell line, HEL [35]. However, α and β*TAC*4 did have limited expression, being specific to immune and megakaryocyte cells [35].

More recently, two splice variants of α*TAC*4 have been identified, α*TAC*4 variant 1 (v1) and v2 [N. M. Page, unpublished observations]. Alternative splicing at the end of

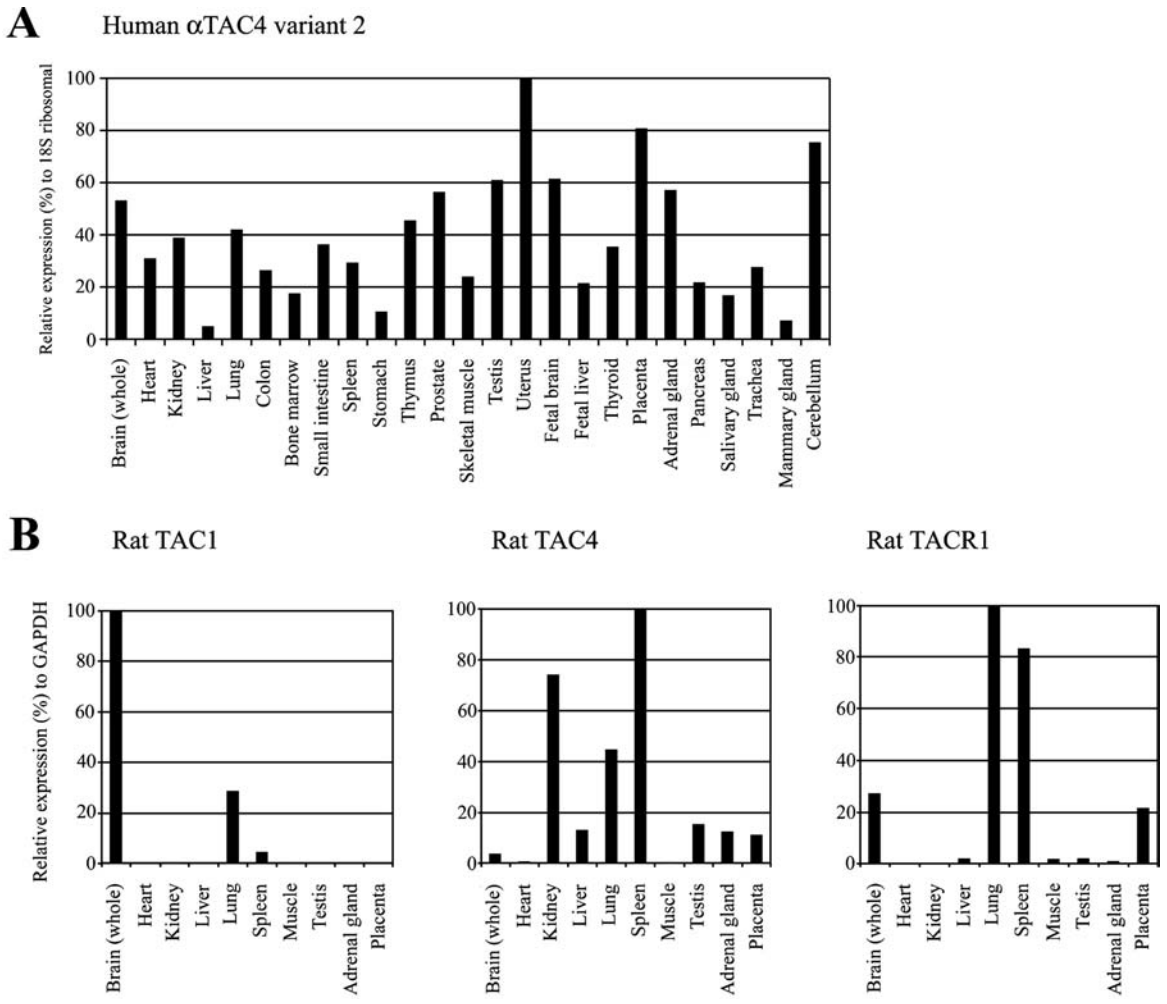

Figure 3. (*A*) Real-time quantitative PCR analysis of the tissue distribution of the *αTAC*4 v2 in 24 human tissues relative to the expression of 18S ribosomal RNA. (*B*) Real-time quantitative PCR analysis of the tissue distribution of *TAC*1, *TAC*4 and *TAC*R1 in 10 rat tissues relative to the expression of rodent GAPDH. The intensities are not a guide to expression between different genes, as the values are relative and not absolute.

exon 1 (fig. 1) produces these. *αTAC*4 v1 is spliced at the same splice donor site utilized in the mouse and rat; in contrast, *αTAC*4 v2 utilizes a premature splice donor site that is common to the human *β*, *γ* and *δTAC*4 [35]. The original strategy for the expression of *αTAC*4 would have reflected the *αTAC*4 v1 population only [35]. Would *αTAC*4 v2 expression be significantly different? The answer is yes. Real-time quantitative PCR for *αTAC*4 v2 reveals ubiquitous expression in a range of human tissues with highest expression in the uterus, placenta, cerebellum and fetal brain (fig. 3 A [Page, unpublished observations]). This indicates that not only is EKB ubiquitously expressed, but would be coexpressed with the tachykinin gene-related peptide, EKC. It can be inferred that *αTAC*4 v2 and *γTAC*4 are the most common and preferred forms of *TAC*4 (fig. 1) and that the splice donor site used in *αTAC*4 v1 (producing EKA) may be essentially redundant in humans.

Peptide localization and processing

The expression pattern for the mRNA of the mouse, rat and human *TAC*4 precursors clearly shows the potential for synthesis and action of the hemokinin and endokinin peptides in a wide variety of cells and tissues. Nevertheless, even though mRNA levels are generally a good reflection of translated protein, they may not always be a good measure of the precise amounts of active peptide, because these will be determined by a number of key factors, including the manner of processing, turnover, storage and secretion. Only one study has reported measuring significant amounts (7.52 fmol/g) of immunoreactive human *γ* and *δTAC*4 precursors present in human term placental extracts using a two-site immunometric assay [35]. However, this assay would not have been able to differentiate between precursor protein and that of the active processed peptide. It remains for specific antibodies to be

developed in order to measure the exact levels of peptides in tissues, to determine their locations by immunocytochemistry and to determine their precise structures and the way they are processed. Indeed, it is clear further work is necessary to determine the precise processing of the human *TAC*4 precursors which do not possess the classical dibasic motif N-terminal to the HK-1-like sequence. Kurtz et al. [34] have hypothesized that in the absence of the N-terminal dibasic cleavage site processing occurs instead on two N-terminal monobasic cleavage sites suggested to occur by nonconsensus and non-proline-directed monobasic processing. Whilst processing on a single monobasic cleavage site has been reported before for NKA(3–10) (i.e. HK^TDSFVGLM-NH_2) (table 1), this system used recombinant vaccinia virus to express *βTAC*1 transfected into AtT-20 cells [38]. It is not known whether this process would occur naturally. In the absence of monobasic processing Page et al. [35] have proposed elongated peptides starting at the end of the leader/signal sequence. It is anticipated that the use of specific antibodies will enable the cleaved precursor fragments to be identified and to determine whether full and efficient processing to biologically active peptides occurs. In this case, *TAC*4 expression in a host of peripheral tissues and cell lines needs to address whether they have the capacity to produce active amidated peptides. Generally, the full posttranslational processing of peptide precursors occurs in specially differentiated endocrine and neuronal cells [40]. However, such proteolytic processing of peptide precursors has been found to be very inefficient in non-endocrine cells [41]. Nevertheless, when non-endocrine cells are presented with a precursor which does not have to be proteolytically processed, that is one terminating in the sequence – GKR, their ability to produce an amidated peptide appears to be a universal feature [40, 41]. C-terminal amidation is important for the agonist activity of the tachykinins [42].

It is highly possible that some earlier reports indicating the presence of SP-like peptides in locations such as endothelial cells [43] and the placenta [44] were caused by the cross-reactivity of anti-SP antibodies with EKA and EKB [35]. Strikingly, a competitive enzyme immunoassay for SP (Bachem, S-1180) is found to display a 100% cross-reactivity with the common C-terminal decapeptide sequence of EKA/B (also rabbit EK-1) and 70% cross-reactivity with HK-1 (fig. 4 [Page, unpublished observations]). Moreover, all of the non-neuronal locations of the tachykinins have concerned mainly SP, the occurrence of which has been established mainly by immunocytochemistry using selective anti-SP antibodies [30]. This calls into question whether these assays were actually measuring SP, HK-1, EK-1, EKA or EKB. It is not known how many different commercially distinct sources of anti-SP antibodies there are, and which part of SP they are specific to. It must surely be a prerequisite to test any anti-SP antibody for cross-reactivity to each of SP, HK-1, EK-1 and EKA/B. Clearly, an antibody that is specific to the N-terminal sequence of each of these peptides will be the most beneficial, as this comprises the most divergent region (table 1). Such a task is challenging, first, because of the small epitope size involved (4–5 amino acids) and second, because it is far from certain what the actual N-terminal sequence of the human endokinin peptides is (or indeed that of HK-1), to use as an antigen.

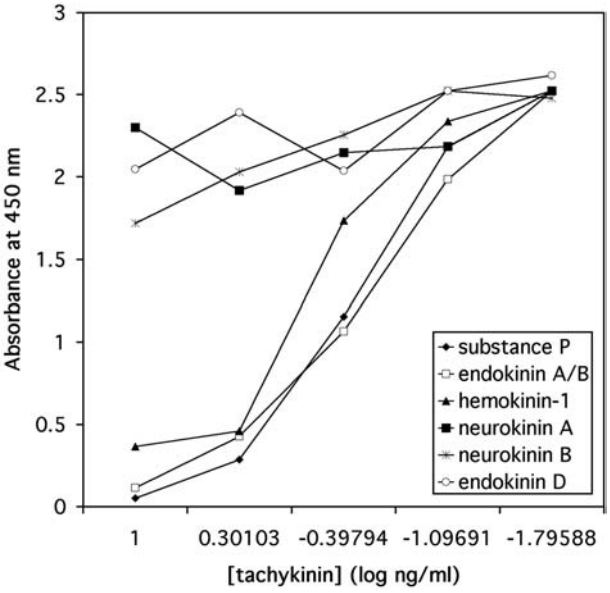

Figure 4. Cross-reactivity of the endokinins, hemokinin-1 and neurokinins with a specific substance P antibody (Bachem, S-1180).

Pharmacology

Zhang et al. [31] first reported that the dose-dependent addition of HK-1 to primary bone marrow cell cultures significantly improved their cell survival and stimulated their proliferation, whereas SP had no effect. Meanwhile, when a selective tachykinin NK_1 receptor antagonist, L-732,138, was added the apoptotic population increased by 15–20%, an effect overcome by the addition of an equimolar concentration of HK-1 and to a lesser extent SP. Thus, it was hypothesized that a novel tachykinin receptor mediated these effects, explaining the differences in the relative potencies of HK-1 and SP.

Ensuing studies have investigated the binding affinity and agonist efficacy of the hemokinin and endokinin peptides at each of the three-mammalian tachykinin receptors. HK-1 has been shown to have equipotent affinity to SP at the human tachykinin NK_1 receptor [34, 35, 37, 45, 46]. In contrast, there is an ~2.8 times greater affinity for HK-1 at the human tachykinin NK_2 receptor than SP [34, 37, 46]; in contrast, HK-1 displays an ~3.8 times lower affin-

ity than SP at the human tachykinin NK_3 receptor [34, 35]. These differences in ligand binding at the NK_2 and NK_3 receptors may be accounted for by the highly divergent N-terminal sequences of these peptides, or the significantly species-dependent variability in the homologies of the NK_2 and NK_3 receptors as compared with the highly species conserved NK_1 receptor. From this latter point, Kurtz et al. [34] and Duffy et al., [37] have shown comparable affinities for HK-1 at the human, mouse and rat NK_1 receptors, yet no study has compared the binding affinities for HK-1 to SP at mouse or rat NK_2 and NK_3 receptors. What are the binding affinities of the human endokinins to the human tachykinin receptors? hHK-1 and hHK-1(4-11) bind to the human NK_1 receptor, with ~14 and 70 times lower affinities, respectively, relative to SP and HK-1 [34]. The C-terminal decapeptide of EKA/B also has ~6.6 times lower affinity compared with SP at the human NK_1 receptor, though this fragment has equipotent affinity at the human NK_2 and NK_3 receptors compared with SP [35]. Interestingly, the EKA/B decapeptide has over a 10 times lower affinity for the uncoupled state of the human NK_1 receptor compared with SP, indicating that the two peptides may bind to a slightly different site on the receptor [35]. This may fit with the theory that there are two agonist-binding conformations of the NK_1 receptor [47]. It must be emphasized, however, that owing to the lack of a N-terminal dibasic cleavage site, the precise sequences of the human endokinin peptides remain unresolved. In this regard, of particular significance is the testing of an extended form of the C-terminal decapeptide sequence of EKA/B, TEAETWEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2, which showed an equipotent affinity for the NK_1 receptor as SP [35]. This indicates that perhaps the correct human endokinin peptide is elongated, but such an extension is compounded by the discovery of rabbit EK-1, which represents the C-terminal decapeptide of EKA/B [N. M. Page, unpublished observations]. The loss of the N-terminal dibasic cleavage site in humans remains a baffling phenomenon.

Similar agonist profiles are observed for SP, HK-1, hHK-1, hHK-1(4–11) and the C-terminal decapeptide of EKA/B at all three tachykinin receptors [34, 37, 45, 46, 48]. In one such analysis, Bellucci et al. [46] employed three different pharmacological assays, rat urinary bladder longitudinal detrusor muscle strips (RUB) for NK_1, rabbit pulmonary artery circular muscle strips deprived of endothelium (RPA) for NK_2 and guinea pig ileum longitudinal muscle myenteric plexus strips (GPI) for NK_3. HK-1 produced full agonist responses as compared with SP, NKA and NKB, which could be antagonized by GR82334, nepadutant and SR142801, respectively. HK-1 was about 3-fold less potent than SP in the RUB assay, while in the RPA and GPI, HK-1 was about 500-fold less potent than NKA and NKB, respectively [46]. Camarda et al. [48] also performed bioassays in their chosen systems for each receptor, rabbit jugular vein (rbJV) for NK_1, rabbit pulmonary artery (rbPA) for NK_2 and rat portal vein (rPV) for NK_3. A comparable result was achieved where HK-1 behaved as a full agonist displaying potencies similar to SP in rbPA and rPV or slightly higher than those of SP in rbJV. The selective NK_1 antagonists SR140333 and MEN11467 antagonized the effects of HK-1 and SP in the rbJV, while in cellular systems, second messenger profiling using calcium mobilization and inositol triphosphate hydrolysis assays generally reflects the results of ligand binding. Again, in experiments including hHK-1 and EKA/B, the greatest potency, equipotent to SP (and about as twice as potent of NKA) is observed at the NK_1 receptor, a phenomenon which is blocked by selective NK_1 receptor antagonists [34, 35, 37]. hHK-1 and EKA/B functionality, similar to SP, is also seen at the NK_2 and NK_3 receptors [34, 37]; in contrast, EKC and EKD are inactive at the NK_1 receptor in calcium mobilization assays [35]. The agonist activity of the latter two ligands has yet to be tested at the NK_2 and NK_3 receptors.

In bioassays, the capacity of HK-1 to induce plasma extravasation and to promote mast cell degranulation has been found to be equipotent to that of SP [31]. Accordingly, the behavior of HK-1 and SP in producing hypotension and salivary secretion in guinea pigs was almost superimposable, suggesting that HK-1 is an equipotent NK_1 receptor agonist in vivo [46]. These cardiovascular effects could be abolished by the structurally unrelated NK_1 receptor antagonists SR140333 and MEN11467 [46]. In rats, there is a complex, but characteristic regional hemodynamic response to SP that is closely mimicked by EKA/B [35]. The striking similarity between the regional hemodynamic effects of these two peptides indicates that EKA/B and SP operate through a common receptor pathway in vivo. The most marked hemodynamic effect of SP and EKA/B is short-lived vasodilatation in the hindquarters vascular bed [35]. In gerbils and mice, in vivo central administration of HK-1 induced foot-tapping and scratching behaviors, respectively, similar to those observed following the central administration of SP and the NK_1 receptor agonist GR73632. The selective NK_1 receptor antagonist MK869 blocked these behavioral effects [37].

The structures of mammalian SP and the SP-like peptides are significantly divergent in their amino acid sequences, particularly at their N-termini (fig. 2C). In contrast, an additional level of divergence exists at the species level between the hemokinins and endokinins (cf. the structure of SP is identical throughout the mammalian kingdom). All of the tachykinins except EKC and EKD possess a predominantly hydrophilic N-terminus and a hydrophobic C-terminus. The C-terminal decapeptide sequence of EKA/B and HK-1 shares the motif FXGLM-NH_2, where X is an aromatic residue (phenylalanine and tyrosine, re-

spectively) which is indicative of a NK_1 receptor-preferring ligand [30]. However, remarkable flexibility within the amino acid residues of the N-terminal moieties of these SP-like peptides exists. Nonetheless, there are consistent features, a neutral hydrophilic amino acid at position 6 (glutamine) and a positively charged hydrophilic amino acid at position 9 (lysine or arginine) from the C-terminus. Such comparisons are useful in seeking clarification as to which amino acids are most crucial for binding at the NK_1 receptor pocket. This will help to determine the effects of the relative contributions of the N- and C-terminal regions of the tachykinin peptides in the binding and activation of the tachykinin receptors.

Is there a novel tachykinin receptor?

Overwhelming evidence attests to the hemokinins and SP-like endokinins exhibiting a remarkable selectivity and potency for the NK_1 receptor similar to SP, with most findings not supporting the speculation for the need for a novel tachykinin receptor. Camarda et al. [48] have stated three lines of evidence for this: first, the order of potency is the same for SP; second, tissues desensitized to SP have reduced responses to HK-1 and third, their effects are blocked by selective NK_1 receptor antagonists. Furthermore, the NK_1 receptor is detected on a variety of peripheral cell lines that ubiquitously express EKA/B and not SP [35]. Nonetheless, it is true that there is a somewhat reduced affinity of the human endokinin peptides at the human NK_1 receptor compared with SP, but this may be a result of incorrect peptide prediction. However, it would be premature to rule out the possibility of an alternative receptor. In fact, a recent study by Grant et al. [49] proposes a novel tachykinin receptor independent pathway for NKB in the mouse. Yet care needs be taken in any study to the selection and interpretation of data for NK receptor antagonists, as remarkable species-related differences in antagonist affinities are inherent. This is owing to the possible interaction of the antagonist with the more variable regions of the rodent and human receptors. More compelling evidence for an alternative receptor comes from the work of Zhang et al. [31] and Zhang and Paige [50], who find contrasting differences in the relative potencies of HK-1 and SP on murine B and T cells, respectively. Despite these findings, no definite conclusions can be drawn. First, NK receptor cross-talk may occur, and as mentioned earlier, the potency of HK-1 at murine NK_2 and NK_3 receptors is unknown, compounded by the fact that NK_2 and NK_3 receptor antagonists were not included in these studies. Second, HK-1 may interact with the NK_1 receptor in a different fashion from SP to activate an alternative signaling pathway. Ultimately, only histological analysis will enable the precise location and association of peptide(s) and receptor(s).

There is significant opportunity for cross-talk between SP, NKA, NKB, HK-1 and the SP-like endokinin peptides with the three tachykinin receptors. However, the essential absence of activity of EKC and EKD at the known receptors, albeit negligible affinity at the NK_3 receptor, would indicate that a fourth receptor does still remain to be elucidated for EKC and EKD. One candidate receptor was the putative human NK_4 receptor. This receptor had a structure similar to the human NK_3 receptor, responded potently to NKB and interestingly was functionally antagonized by the endogenous opioid peptide dynorphin [51]. A subsequent investigation of the human NK_4 receptor has unfortunately found this receptor to be the guinea pig NK_3 receptor [52]. Hence, the search for a receptor continues, with the possibility that interaction may occur with an already known receptor subtype. Nonetheless, the interaction of opioids with the tachykinin receptors is appealing, considering the close proximity and relationship of tachykinin and opioid nerve fibres [53]. It is interesting to note that some analogues of SP have been found to bind to opioid receptors [54, 55] and that some candidate NK_1 receptor antagonists also show significant binding affinity to the human mu-opioid receptor [56]. An analogue of SP with equalized lipophilicity of the N- and C-terminal regions (cf. EKC and EKD) was found to be antagonized by naloxone following injection into mice, while SP was not displaced [54]. Yet in preliminary experiments in vitro neither EKC nor EKD were able to displace naloxone from opioid receptors [N. M. Page, unpublished observations]. For the present, their species-specific nature and the lack of a candidate receptor limits their further characterization, though the discovery of rabbit EK-2 opens up prospects for an animal model.

Perspective

In mice, rats and humans, *TAC*4 is widely distributed, with expression levels and sites distinct from that of *TAC*1, particularly within the peripheral tissues. The peripheral expression of *TAC*4 is therefore particularly intriguing, leading us to propose that HK-1 and EKA/B are the peripheral SP-like endocrine/paracrine agonists where SP is not expressed. Persuasive is that they may play a fundamental role in cellular physiology, whereby, via NK_1 receptors, they interact in an autocrine or paracrine manner. Definitely, their mode of action seems to be endocrine rather than neuronal. In this regard, it is interesting that SP has been shown to be mitogenic and a potent stimulator of fibroblast proliferation [57]. Additionally, in the absence of nerves, the local production of EKA/B by tissues such as the placenta and endothelium devoid of SP could have particular importance in determining regional blood flow such as that of the placental/uterine circulation [35]. In the adrenal gland, which

has the potential to express all four endokinins, it is noteworthy that SP treatment enhances aldosterone production by eliciting catecholamine secretion, while there are indications that SP stimulates the maintenance of normal growth and steroidogenic capacity, playing an important role in the stimulation of adrenal growth during fetal life [58]. However, to date, the most substantial evidence supporting a physiological role for the endokinin group is for the role of HK-1 in the regulation and developmental steps of B [31] and T cell lymphopoiesis [50].

Perhaps one of the most challenging future aspects will be dissecting a distinct role for the endokinin group from that of SP. For instance; Why is SP expression limited to the central and peripheral nervous system and certain immune cells? Is it the constraints of its promoter that explain the evolution of a second, peripheral NK_1 receptor ligand? The site and structural elements of the *TAC*4 promoter remain to be elucidated. Could the divergent sequences of EKA/B and HK-1 offer a mechanism by which the metabolism of these peptides is differentially controlled, either by their resistance to proteolytic enzymes or by their degradation, allowing generation of novel bioactive metabolites? Many studies have now shown that the N-terminal fragments of SP, particularly SP(1–4) [59] and SP(1–7) [60] are biologically active SP metabolites and that the formation of these products can be blocked by the action of specific endopeptidase inhibitors [61]. Such answers may enable new insights and leads into the development of novel therapeutic targets, into many of the conditions formerly attributed to SP, especially those in the periphery, such as rheumatoid arthritis, vasodilatation, inflammation, irritable bowel syndrome, pancreatitis, cancer and asthma.

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