very rapid rate. Alternatively, it is possible that a feedback system exists such that incomplete degradation accelerates autophagocytosis. However, with the exception of the Purkinje cells, we detected no obvious disturbances in the ultrastructure of neurons containing ceroid-lipofuscin. This would not be expected if an increased rate of organelle breakdown occurred unless it were balanced by an equivalent change in the rate of synthesis.

These results may have implications for the etiology of the group of inherited neuronal ceroid lipofuscinoses (NCL) (*3*). Not only do the granular and curvilinear profiles of the leupeptin-induced ceroid-lipofuscin closely resemble those found in patients with NCL diseases, but also, in both leupeptin-treated rats and patients there is a focal degeneration of Purkinje cells in the cerebellum. Perhaps the accumulation of pigment in nerve cells of individuals with NCL is caused by a defective (or absent) lysosomal proteinase, in much the same way that defective lipases, galactosidases, or other enzymes cause the accumulation of various substances in the hyperlipoproteinemias, Tay-Sachs disease, or other inherited lysosomal storage disorders (*18*).

Finally, our findings suggest a means for studying the functional consequences of dense body accumulation. Since leupeptin is a reversible inhibitor, the drug is probably removed from the brain once the osmotic pump has been emptied. By comparing differentially affected cell populations in the same animal (during and after drug treatment) as well as in animals exposed to different concentrations of drugs, it should be possible to arrive at correlations between the accumulation of lipofuscin and various structural and functional measures. These approaches, coupled with recent evidence of biochemical changes associated with ceroid-lipofuscin accumulation (*19*), could lead to the development of experimentally accessible models for studying the role of dense body accumulation in NCL diseases and aging.

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11. For electron microscopy, animals were perfused with a fixative containing 2.5 percent each of paraformaldehyde and glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.2. Portions of these brains were used for light microscopy; additional animals were perfused with 10 percent buffered Formalin for light microscopy.
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Novel Pharmacology of Substance K–Binding Sites: A Third Type of Tachykinin Receptor

Abstract. *The tachykinins are a family of peptides with the carboxyl terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. Three major mammalian tachykinins have been identified—substance K, neuromedin K, and substance P—but only two tachykinin receptors have been postulated. Three tachykinins were labeled with radioiodinated Bolton-Hunter reagent and their binding characteristics were determined in crude membrane suspensions from several tissues. In cerebral cortex labeled eledoisin exhibited high-affinity binding that was inhibited by tachykinins in a manner indicating a definitive SP-E receptor site. In gastrointestinal smooth muscle and bladder, high-affinity binding of labeled substance P was inhibited in a pattern indicating a definitive SP-P site. In intestinal smooth muscle and bladder, however, labeled substance K and labeled eledoisin were both bound in a pattern indicating a preference for substance K itself. The results suggest the existence of three distinct types of tachykinin receptors: SP-P, SP-E, and SP-K.*

The tachykinin family of peptides is characterized by the carboxyl terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. The molluscan salivary gland peptide eledoisin and the amphibian skin peptides physalaemin and kassinin are members of this family, as is the mammalian putative neurotransmitter substance P (*1*).

In addition to substance P, two novel tachykinins have recently been identified in mammals. Neuromedin K (also called neurokinin β) has been isolated from spinal cord (*2, 3*), as has a kassinin-like neuropeptide named substance K (also called neurokinin α) (*2, 4*). Nawa *et al.* (*5*) used bovine striatal messenger RNA and bacterial cloning to identify a substance P precursor molecule, α-preprotachykinin, which contains only one copy of substance P, and a second precursor, β-preprotachykinin, which con-

tains one copy each of substance P and substance K.

Despite the existence of three major mammalian tachykinins, only two different tachykinin receptors have been postulated. On the basis of the sensitivity of smooth muscle preparations to contraction and tachyphylaxis elicited by the various tachykinins, it has been proposed that there is an SP-P receptor at which most tachykinins are essentially equipotent and an SP-E receptor at which eledoisin and kassinin are substantially more potent than substance P and physalaemin (*6*). Substance P is generally accepted as being the endogenous ligand for the mammalian SP-P receptor, while it has been proposed that substance K is the endogenous ligand for the SP-E receptor (*7*). Ligand binding assays involving radioactively labeled tachykinins have generally confirmed these receptor designations. Mammalian brain, salivary gland, and gastrointestinal smooth muscle contain specific binding sites that are analogous to SP-P receptors since they have a higher affinity for substance P and physalaemin than for other tachykinins (*8, 9*). The cerebral cortex also contains an SP-E receptor binding site that has a preferential affinity for eledoisin, kassinin, and neuromedin K compared to substance P and physalaemin (*10*). Using autoradiography, we have already shown that substance P and substance K bind to gastrointestinal smooth muscle at different sites (*11*). In this report we provide evidence that labeled substance K binds to a third type of tachykinin-binding site in gastrointestinal smooth muscle and urinary bladder membranes. The site has a preference for substance K over all other tachykinins and occurs in tissues previously thought to contain primarily an SP-E receptor.

Table 1. Rank order of tachykinins in inhibiting binding of labeled tachykinin in membrane suspensions from various tissues. Assay methodology was as described in the legend to Fig. 1. The IC_{50} (nanomolar; mean of two to four determinations) is shown in parentheses for each peptide. Abbreviations: SK, substance K; Kass, kassinin; Ele, eledoisin; NK, neuromedin K; SP, substance P; and Phy, physalaemin.

BHSK						BHE						BHSP, rat duodenum‡	
Rat duodenum*		Mouse bladder*		Guinea pig intestine*		Rat duodenum*		Mouse bladder*		Rat cortex†			
SK	(1)	SK	(1)	SK	(1.5)	SK	(0.5)	SK	(0.5)	NK	(2)	SP	(0.3)
Kass	(11)	Kass	(8)	Kass	(12.5)	Kass	(1.5)	Kass	(0.5)	Kass	(4)	Phy	(0.5)
Ele	(20)	Ele	(9)	Ele	(15)	Ele	(2.5)	Ele	(4)	Ele	(4)	SK	(5)
NK	(40)	NK	(15)	NK	(70)	NK	(6.5)	NK	(12.5)	Phy	(22)	Ele	(8)
SP	(100)	SP	(125)	SP	(130)	SP	(15)	Phy	(17.5)	SK	(38)	Kass	(9)
Phy	(200)	Phy	(175)	Phy	(325)	Phy	(40)	SP	(50)	SP	(100)	NK	(45)

*SP-K receptor binding pattern. †SP-E. ‡SP-P.

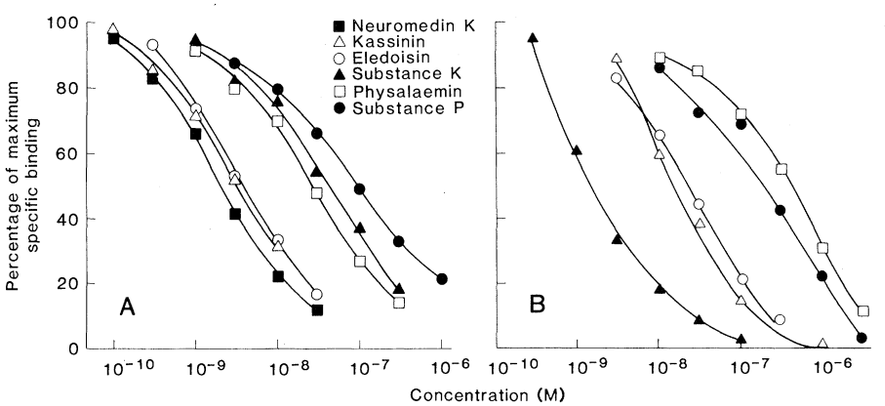

Fig. 1. Competitive inhibition by tachykinins of BHE binding in rat cortical crude membranes (A) and BHSK binding in rat duodenal smooth muscle membranes (B). All tissues were used fresh except mouse bladders, which were stored at −80°C until use. Mucosa and submucosa were scraped off rat duodenums with a scalpel and the remaining smooth muscle was used. Longitudinal smooth muscle was obtained from the entire guinea pig small intestine by rubbing with a cotton swab. Tissues were homogenized with a Polytron in ice-cold 50 m*M* tris-HCl (*p*H 7.4) containing 120 m*M* NaCl and 5 m*M* KCl. The 48,000*g* pellet was resuspended for 30 to 60 minutes at 4°C in 50 m*M* tris-HCl (*p*H 7.4) containing 300 m*M* KCl and 10 m*M* EDTA with periodic mixing. After centrifugation as above the pellet was resuspended and washed twice in 50 m*M* tris HCl (*p*H 7.4) at 4°C. The washed pellet was resuspended and a portion was incubated for 90 minutes (equilibrium time for BHSK and BHSP) or 150 minutes (equilibrium time for BHE) at 25°C in 50 m*M* tris-HCl (*p*H 7.4) containing bovine serum albumin (BSA; 400 μg/ml), bacitracin (40 μg/ml), leupeptin (4 μg/ml), chymostatin (4 μg/ml), and $MnCl_2$ (3 m*M*) in a final volume of 0.5 ml in polypropylene tubes (*9*). BHSK, BHE, or BHSP, prepared and purified as previously described (*14*), was included at a concentration of 100 p*M*. The final concentration of tissue (calculated on a wet weight basis) was 3 percent for all tissues except bladder (8 percent). All unlabeled peptides were stored as stock solutions at −20°C in dilute acetic acid containing 1 percent β-mercaptoethanol and were diluted in incubation buffer for the assays. The assays were terminated by rapid addition of 3.5 ml of 50 m*M* tris-HCl (*p*H 7.4) containing 3 m*M* $MnCl_2$ at 4°C and filtration over Whatman GF/B filters that had been soaked for 12 to 24 hours in 0.5 percent BSA (for BHE and BHSK) or 0.1 percent polyethylenimine (for BHSP) at 4°C. The filters were washed twice more in a similar manner and then counted in a gamma counter. Termination of assays and washing were completed in less than 30 seconds. Specific binding [the difference between binding in the absence and binding in the presence of 1 μ*M* unlabeled substance K (for BHSK), eledoisin (for BHE), or substance P (for BHSP)] constituted 70 to 80 percent of total binding. Each experiment was carried out two to four times. A typical experiment is depicted in each panel.

Rat duodenum and mouse bladder have been reported to contain primarily SP-E receptors on the basis of experiments on isolated smooth muscle preparations (*6*). We examined the pharmacology of [^{125}I]Bolton-Hunter reagent–labeled eledoisin (BHE) binding in crude membrane suspensions from these tissues. Although BHE bound to both tissues, unlabeled eledoisin was not the most potent tachykinin in inhibiting the binding. Rather, substance K was, with a median inhibitory concentration (IC_{50}) of 0.5 n*M* in both duodenal smooth muscle and bladder; eledoisin was five to ten times less potent (Table 1). The competition rank order for the tachykinins in these tissues was substance K > kassinin > eledoisin > neuromedin K > substance P <> physalaemin (Table 1), indicating that BHE was not binding to a definitive SP-E receptor site (*10*).

To confirm that BHE is capable of binding to SP-E sites, we examined BHE binding in crude membrane suspensions from rat cerebral cortex. It was previously observed (*10*) that in rat cortical synaptosomes BHE binds to SP-E sites that have a preferential affinity for eledoisin, kassinin, and neuromedin K. In cortical membranes we found that the competition rank order for BHE binding was neuromedin K (IC_{50}, 2 n*M*) > kassinin = eledoisin > physalaemin > substance K > substance P (Fig. 1A). This rank order is in agreement with previous findings (*10*) and is markedly different from the rank order of tachykinins in inhibiting BHE binding in duodenum and bladder (Table 1).

We also investigated the binding of [^{125}I]Bolton-Hunter reagent–labeled substance P (BHSP) to smooth muscle membranes from rat duodenum and mouse bladder. Consistent with BHSP labeling an SP-P site, the competition rank order in both tissues was substance P (IC_{50}, 0.3 n*M*) > physalaemin >> substance K > eledoisin > kassinin > neuromedin K (Table 1). This pattern of inhibition is in agreement with previous characterizations of BHSP and tritiated substance P binding to SP-P sites in the central nervous system, salivary glands, and gastrointestinal smooth muscle (*8, 9*) and indicates that the duodenum and bladder contain SP-P sites.

The binding characteristics of substance K were examined directly with [^{125}I]Bolton-Hunter reagent–labeled substance K (BHSK). In duodenum and bladder tissue unlabeled substance K was the most potent inhibitor of BHSK binding, with an IC_{50} of 1 n*M* in both cases. The competition rank order of tachykinins in inhibiting BHSK binding was substance K >> kassinin > eledoisin > neuromedin K > substance P > physalaemin (Fig. 1B and Table 1). This pattern is nearly identical to that for inhibition of BHE binding in these tissues (Table 1), but clearly differs from that for BHE binding to SP-E sites in cerebral cortex (Fig. 1A). We also observed that BHSK binding to guinea pig intestinal smooth muscle membranes showed this preference for substance K (Table 1). Most of the tachykinins were more potent in inhibiting BHE binding in duodenum and bladder than in inhibiting BHSK binding in these tissues, perhaps indicating that BHE binds to more than one site. Nevertheless, in duodenum and bladder both ligands bound primarily to the same novel site. This site has a high affinity for substance K (dissociation constant, approximately 3 n*M*) and is not an SP-E or SP-P site. On the basis of these results, we propose that a third type of tachykinin receptor exists in peripheral tissues. This receptor is distinct from SP-P and SP-E receptors and should be designated the SP-K receptor.

Extrapolations between ligand binding data and bioassays in preparations of intact tissues are confounded by several factors. In intact tissues, such factors as metabolism, access to receptors, desensitization, and differential affinities for and densities of multiple receptor types mediating the same biological response can complicate results with tachykinin peptides (*12*). On the other hand, binding assays suffer from the use of disrupted tissue in nonphysiological conditions to measure what can be nonfunctional binding sites. These limitations notwithstanding, for the tachykinins two distinct types of mammalian binding sites have previously been identified that exhibit remarkable similarity to the two types of receptors postulated in intact tissues, SP-P and SP-E (*6, 8–10*). The results presented here clearly indicate that a third type of tachykinin binding site exists in the periphery, and we believe that this indicates the existence of a third type of receptor, the SP-K receptor. Our characterization of BHSK binding in the rat duodenum is in agreement with the observation (*7*) that substance K is moderately more potent than kassinin and 100 times as potent as substance P and physalaemin in producing contraction of the rat duodenum (Fig. 1B and Table 1).

Other investigators have suggested the existence of multiple types of tachykinin receptors in the periphery (*6*). Using BHSK, we have shown that an SP-K receptor exists in peripheral tissues in addition to the SP-P receptor. In rat duodenal smooth muscle and mouse bladder we have been unable to demonstrate an SP-E receptor, although this receptor is found in the rat cerebral cortex. Tissues previously classified as bearing SP-E receptors (*6*) may all contain SP-K and SP-P receptors rather than definitive SP-E receptors. In these tissues, in which eledoisin and kassinin are up to 150 times more potent than substance P and physalaemin, it is possible that there are many more SP-K receptors than SP-P receptors. The existence of a very minor population of SP-E receptors cannot be completely ruled out.

Biological assay data indicate that substance P, physalaemin, eledoisin, and kassinin are all equipotent in SP-P tissues. However, competitive binding of labeled substance P to membranes clearly shows that the SP-P binding site has much greater affinity for physalaemin and substance P than for eledoisin and kassinin. Eledoisin and kassinin have nearly the same potencies in their inhibition of BHSP binding as in their inhibition of BHSK binding (Table 1). Thus, in tissues containing similar numbers of SP-K and SP-P receptors, substance P may cause contraction through SP-P receptors, whereas eledoisin and kassinin may act through both SP-K and SP-P receptors. This could give the appearance of an SP-P tissue.

The three distinct binding patterns for the tachykinins indicate three separate receptor systems: SP-P, in which physalaemin and substance P are most potent; SP-K, in which substance K is most potent; and SP-E, in which kassinin, eledoisin, and neuromedin K are most potent. In mammals this suggests that the endogenous ligands for these receptors are substance P, substance K, and neuromedin K, respectively. The likely neuronal coexistence of the two related neuropeptides, substances P and K, acting on two distinct receptor systems, is analogous to the situation that has been described for the mammalian opioid peptides (*13*). Substance K may have important physiological roles outside the mammalian central nervous system.

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