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An electrophysiological investigation of the effects of cholecystokinin on enteric neurons

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Irma W. M. Schutte

Proefschrift

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Cholecystokinin (CCK) is a peptide, which is present in the gastrointestinal tract in endocrine cells and in the enteric nervous system (ENS). A possible function in the control of motility of the small intestine has been attributed to neuronal CCK. The aim of this thesis was to obtain a fundamental insight into the action and effects of CCK on enteric neurons. Therefore, intracellular recordings were made of myenteric neurons in an isolated preparation of the guinea-pig ileum. Two types of excitable myenteric neurons were distinguished. Neurons in which the action potential showed a pronounced inflexion (shoulder) on the falling phase, were classified as AH neurons, the others as S neurons. The effects of CCK-8 and CCK-8NS on both types of neurons were determined. Application of CCK evoked dissimilar excitatory effects in the two types of neurons, which presumably are related to the function of these neurons in the ENS. Application of CCK evoked excitatory effects on almost all S neurons (inter- or motor-neurons). The effect was mediated by both CCK_A and CCK_B receptor subtypes and was different for both receptor subtypes with respect to action in time. Some S neurons possessed exclusively the CCK_A or the CCK_B receptor subtype, but others possessed both subtypes. The predominant effect of CCK on AH neurons (sensory neurons) was also slow excitation. These AH neurons were all endowed with both CCK receptor subtypes. The CCK_A and CCK_B receptor subtypes on AH neurons had not only a different affinity for CCK, but also mediated the effects through different ionic channels. Results of experiments in which the effects of CCK antagonists on synaptic transmission were determined, showed that CCK in the ENS has besides a hormonal function, a function as neurotransmitter.

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Stellingen

1. Het gebruik van een eenduidige electrofysiologische indeling van enterale neuronen, maakt het interpreteren van literatuur gegevens een stuk eenvoudiger. *dit proefschrift*

2. De electrofysiologische indeling in myenterische AH en S neuronen heeft een functionele betekenis. *Bornstein (1994) J Auton Nerv Sys 48:1-15; dit proefschrift*

3. Cholecystokinine (CCK) is een neurotransmitter betrokken in de regulatie van de dunne darm motiliteit. *dit proefschrift*

4. Men dient eerst meer te weten over de exacte functie van de CCK receptoren in het maagdarmkanaal, alvorens CCK antagonisten gericht worden toegepast voor behandeling van functionele darmstoornissen.
Wettstein et al., (1994) Pharmacol Ther 62: 267-282; dit proefschrift

5. De betrouwbaarheid van de xylose absorptie test voor het vaststellen van malabsorptie bij mens en dier dient sterk in twijfel te worden getrokken, omdat een belangrijk deel van xylose in het maagdarmkanaal wordt gemetaboliseerd.
proefschrift J.B. Schutte (1991), Wageningen

6. Het feit dat het anti-nutritionele effect van visceuze wateroplosbare niet-zetmeel koolhydraten bij vleeskuikens afhankelijk is van de vetbron in het rantsoen, duidt er op dat de viscositeit alleen niet de verklarende factor is voor het anti-nutritionele effect. *D.J. Langhout et al., (1997) Br Poultry Sci 38(5)*

7. Onnodig gebruik van antibiotica door mensen kan wel eens een groter gevaar voor de volksgezondheid vormen dan het antibioticum gebruik in veevoeders.

8. Het gebruik van homeopathische middelen wordt vaak op de foutieve veronderstelling "baat het niet, schaadt het niet" gebaseerd.

9. Tegen de tijd dat een kind de aanbevolen leeftijdsgrens voor gebruik van speelgoed heeft bereikt, is het kind vaak niet meer geïnteresseerd in het betreffende speelgoed.

10. Pas als je eigenlijk van alles zou moeten doen, kun je genieten van niets doen.

11. Door grootouders een belangrijke bijdrage te laten leveren in de opvoeding van de kleinkinderen, kan het tekort in de reguliere kinderopvang vrijwel geheel opgeheven worden.

12. Men hoeft geen materiedeskundige te zijn om het proefschrift van een vakidioot te kunnen corrigeren. *Een leek*

Stellingen behorende bij het proefschrift:
"An electrophysiological investigation of the effects of cholecystokinin on enteric neurons"

Irma W.M. Schutte
Wageningen, 21 januari 1998.

Voor Pim, Nicole en Sylvie

Voor mijn ouders

Contents

Chapter 1

General introduction

General introduction

The enteric nervous system (ENS) is a local neural network within the wall of the gastrointestinal tract that coordinates the activity of various gastrointestinal functions (Wood, 1994*a*). In the gastrointestinal tract, complex reflex activities involving gastrointestinal motility, mucosal blood flow and intestinal ion transport occur in the absence of input from the central nervous system (CNS), implying that the neural circuitry for such behavior is found entirely within the ENS (Bayliss and Starling, 1899; Gershon, 1980). Therefore, sensory receptors, sensory neurons, interneurons and motorneurons are all present in the ENS. In addition, the intestine receives inputs from the central nervous system via sympathetic and parasympathetic nerves and returns sensory information via vagal and spinal afferent neurons.

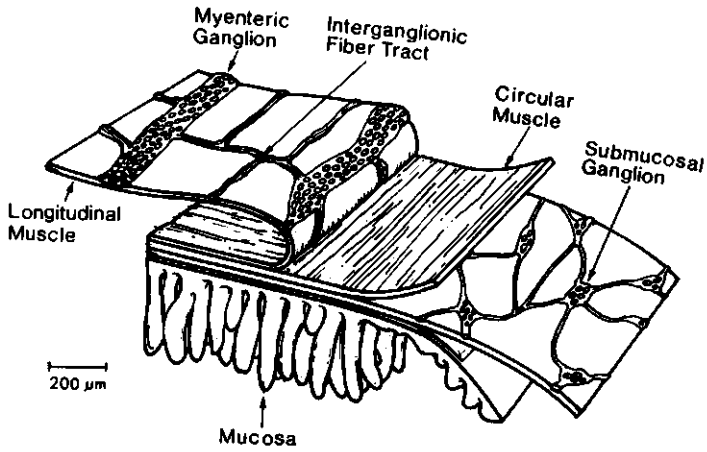

Figure 1. Schematic drawing of the wall of the small intestine showing the arrangement of the enteric plexuses (from Wood, 1994a).

Practically all the cell bodies of enteric neurons are found in small ganglia in two plexuses: the myenteric plexus and the submucous plexus (Fig. 1). These networks of neurons and their interconnections lie between the longitudinal and circular muscle layers and between the circular muscle and mucosa, respectively. The plexuses form a continuous network from the upper oesophagus to the internal anal sphincter. Most of the neural circuitry responsible for the control of motility lies within the myenteric plexus (Bornstein, 1994). The submucosal neurons are primarily

involved with modulation of the gastrointestinal blood flow and intestinal ion transport (Surprenant, 1994). Each ganglion contains from 10 up to 100 neurons and in total there are about 10^8 neurons in the ENS (Furness *et al.*, 1990).

The guinea-pig gastrointestinal tract has been the principal model for investigation of elements of the ENS. Important information on properties and functions of enteric neurons can be obtained by electrophysiological methods of recording and by histochemical techniques. Physiological and microscopic analysis of neural circuits is facilitated because the plexuses form flat sheets in the opened intestine. These plexuses can be exposed for physiological recording, while their connections with other neurons and effector tissues are retained. Most studies are performed on the myenteric plexus of the guinea-pig small intestine. The diversity of enteric neurons in the ENS has become apparent in functional, pharmacological, morphological, electrophysiological and neurochemical studies and the neurons can be classified accordingly. It should be noted that, although the small intestine of the guinea-pig has been studied to a far greater extent than any other region of any species bowel, it is not yet apparent how typical the guinea-pig small intestine actually is.

Electrophysiologically, various classes of enteric neurons have been described (Wood, 1994*a*). The various classes are differentiated on the basis of their excitability level and the degree and nature of synaptic input. The two main excitable cell types are S neurons receiving fast synaptic input and AH neurons showing a longlasting after-hyperpolarization (Nishi and North, 1973; Hirst *et al.*, 1974). A main difference between AH and S neurons is that Ca^{2+} as well as Na^+ carries part of the current of action potentials in AH neurons, while only Na^+ carries this current in S neurons (Hirst *et al.*, 1985; Wood, 1994*a*). The Ca^{2+} component of the action potential in AH neurons is responsible for a pronounced shoulder on the falling phase of the action potential, which is not encountered in S neurons (Hirst *et al.*, 1985). Between different research groups, there are however large differences between selection criteria to distinguish the cell types and these criteria do not necessarily divide neurons into identical classes (Bornstein *et al.*, 1994; Wood, 1994*b*). Many electrophysiological features of the myenteric neurons of the small intestine can be generalized to other parts of the guinea-pig intestinal tract (Schemann and Wood, 1989; Tamura and Wood, 1989), and to other species (Brookes *et al.*, 1987; Furakawa *et al.*, 1986; Cornelissen *et al.*, 1996; Thomsen *et al.*, 1997).

The use of intracellular dye injection methods has allowed the electrophysiological properties of neurons to be correlated with their morphology. AH

neurons appear to have large smooth cell bodies with two or more long processes. Most S neurons are smaller and have several short processes and only a single long process (Furness *et al.*, 1988; Iyer *et al.*, 1988).

The myenteric neurons can also be classified into functional neuronal types. A wide variety of physiological and pharmacological evidence indicates that the myenteric nervous system contains sensory neurons, orally directed interneurons, anally directed interneurons and motor neurons supplying the circular and longitudinal muscle. There are both inhibitory and excitatory motor neurons (Bornstein, 1994) supplying gastrointestinal smooth muscle. Electrophysiological properties of myenteric neurons are shown to be correlated with their function. S neurons are shown to be inter- or motor-neurons (Bornstein *et al.*, 1991; Brookes *et al.*, 1992). Among S neurons, electrophysiological characteristics that distinguish between motor- and interneurons are yet to be defined. AH neurons are proposed to be sensory neurons (Song *et al.*, 1991; Bornstein, 1994; Kunze *et al.*, 1995).

The diversity of the enteric neurons becomes however most evident when the neurons are classified according to their content of established or putative neurotransmitters. More than 25 neurotransmitter candidates have been discovered in the gastrointestinal tract (McConalogue and Furness, 1994). Many of these substances are found in both the ENS and the brain. For acetylcholine, norepinephrine, 5-HT and substance P a neurotransmitter function has been demonstrated in the ENS (Wood, 1994*a*). Putative enteric neurotransmitters include peptides, amino acids and nitric oxide. Enteric neurons always contain several substances that participate in neurotransmission and some contain four or more potential transmitters (Costa *et al.*, 1987; Furness *et al.*, 1992). For many neurons one substance seems to have a major role in transmission, while other substances have a subsidiary or modulatory role (Furness *et al.*, 1992).

By far the largest single group of potential transmitters in the ENS is composed of the peptides. More than 15 different peptides are found in the ENS, which subserve a variety of roles. They can act as primary transmitters, but often act as co-transmitters in enteric neurons and have a neuromodulatory role, for example by influencing transmitter release or by causing long-term changes in excitability (Furness *et al.*, 1992).

The peptides localized to gastrointestinal neurons, are all found elsewhere in the body in neurons (notably in the brain) or in endocrine cells (Dockray, 1994). The physiological roles of neuropeptides are therefore often difficult to define. For instance, the responses evoked by exogenous application of a peptide may reflect

actions normally exerted in paracrine, endocrine, or neurotransmitter regulated systems. Also, in studies of release mechanisms it is often difficult to distinguish material derived from neurons and endocrine cells. A peptide that besides a hormonal function, might function as a neurotransmitter in the gastrointestinal tract is cholecystokinin.

Cholecystokinin

Cholecystokinin (CCK) is a member of a family of related peptides first isolated in the gastrointestinal tract and subsequently identified in the brain (Williams, 1982). This family of peptides exhibits diverse functions on target tissues. In the CNS, CCK is a neurotransmitter involved in memory processes, pain perception and elicitation of anxiety and satiety (Crawley and Corwin, 1994; Liddle, 1994). In the periphery, CCK is known to delay gastric emptying, stimulate pancreatic enzyme secretion, contract the gallbladder, and enhance intestinal motility (Walsh, 1994). There are several different molecular forms of CCK that vary in chain length by extension at the N-terminus. The major form found in central and peripheral neurons is the sulfated octapeptide, CCK-8, and is the minimum sequence for biological activity in the periphery of rodents (Walsh, 1994). The larger forms of 22, 33, 39 and 58 residues all terminate in CCK-8 and are the predominant forms found in gut endocrine cells (Crawley and Corwin, 1994).

Two CCK receptor subtypes have been identified, and these are referred to as CCK_A (alimentary) and CCK_B (brain) receptors. Both receptors occur in the periphery and in the CNS, although CCK_A receptors are more predominant in the periphery, notably in the gastrointestinal tract and CCK_B receptors are more numerous in the brain. Several different classes of receptors antagonists have been developed for CCK receptors, that have excellent selectivity and high affinity for either CCK_A or CCK_B receptors (Liddle, 1994).

In the gastrointestinal tract, CCK like peptides have been localized not only in endocrine cells, but also in the ENS. The highest concentrations of CCK are found in the mucosa of the duodenum and proximal jejunum where it is contained in a specific class of gut endocrine cells (Polak *et al.*, 1975; Buffa *et al.*, 1976). CCK is released from endocrine cells in response to a meal. The physiological plasma concentration of CCK peptides in mammals is approximately 1 pM, increasing to about 10 pm during maximal physiological stimulation, such as the ingestion of a

meal (Rehfeld, 1989; Walsh, 1994). In the ENS, CCK immunoreactive nerve fibers are found in the small intestine and the colon (Larsson and Rehfeld, 1979: Schultzberg *et al.*, 1980; Leander *et al.*, 1984; Furness *et al.*, 1984). It is therefore difficult to distinguish between hormonal and neural effects. An example of purely hormonal activity is stimulation of gallbladder contraction (Mawe, 1991), while most of the other actions seem to involve a combination of hormonal and neural pathways or to be entirely neural. The effects of CCK may be mediated through muscularly located receptors as well as through receptors present on neurons.

In the guinea pig ileum, CCK evokes contraction of the longitudinal and circular muscles by stimulating cholinergic (Vizi *et al.*, 1973) and substance-P containing myenteric neurons (Hutchinson and Dockray, 1981; Bartho *et al.*, 1983). These effects are probably responsible for the potent stimulant action of CCK-8 on the peristaltic reflex (Bartho *et al.*, 1983), as distension of the ileum has been shown to evoke CCK release (Donnerer *et al.*, 1985). Electrophysiological recordings have shown that CCK evokes mainly slow excitatory responses in myenteric neurons of the guinea pig (Nemeth *et al.*, 1985).

Presently, selected CCK_A and CCK_B antagonists are being examined in man for their therapeutic usefulness in mental and digestive disorders (Wettstein *et al.*, 1994). CCK antagonists are potentially useful for the treatment of functional bowel disorders, such as dyspepsia and irritable bowel syndrome. More research is however needed for a better understanding of CCK function in the gastrointestinal tract. The knowledge about the function of CCK as a neurotransmitter in the ENS is scarce and the precise mode of action of CCK on the neurons is not well known. Further, no information is available about the CCK receptor subtypes in the ENS.

Aim and outline of the thesis

The aim of the research is to obtain a fundamental insight in the mechanisms by which CCK influences the nervous system in the gastrointestinal tract. With this aim intracellular recordings are made of myenteric neurons in isolated preparations of the guinea pig distal ileum.

To determine the direct effects of CCK on neuronal functioning, the effects of CCK on the two different neuron types, S and AH neurons, are studied. The CCK receptor subtypes involved in the CCK-induced effects are characterized and the resistance changes through which CCK evokes its action on the different cell types

and different CCK receptor subtypes are investigated.

Also the role of CCK as neurotransmitter in the ENS is investigated. CCK is though to be a putative neurotransmitter involved in the generation of slow synaptic excitation (Wood, 1994*a*), based on the presence of CCK in myenteric neurons (Larsson and Rehfeld, 1979: Schultzberg *et al.*, 1980; Leander *et al.*, 1984; Furness *et al.*, 1984) and its slow excitatory action on neurons (Nemeth *et al.*, 1985).

To answer these questions regarding the actions of CCK on enteric neurons the following outline is used:

1. To distinguish enteric AH and S neurons unequivocally, the possibility of electrophysiologically identifying the enteric AH and S neurons "on line" by use of one single criterion, namely the presence of a shoulder on the action potential, is examined.

2. The actions of CCK on the myenteric S and AH neurons are investigated. The receptor subtypes involved are characterized and the action of CCK on each of the receptor subtypes is investigated. Also, the resistance changes mediated by the CCK receptor subtypes are investigated.

3. The action of CCK as a putative neurotransmitter is investigated, by determining the effects of selective CCK antagonists on electrically evoked slow synaptic excitation.

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Chapter 2

Somal size and location within the ganglia for electrophysiologically identified myenteric neurons of the guinea-pig ileum

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Somal size and location within the ganglia for electrophysiologically identified myenteric neurons of the guinea-pig ileum

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Abstract

The main goal of the present study was to examine the possibility of electrophysiologically identifying the excitable enteric S and AH neurons by use of one single criterion. Intracellular recordings were made from 189 cells of 64 ganglia in isolated preparations of the myenteric plexus of the guinea-pig distal ileum. The recordings were made under visual control of the cells by using Hoffman Modulation Contrast optics at high magnification (600 x). From photomicrographs the soma size and the location within the ganglion of the individual (unstained) cells were determined. The cells were classified into three types according to their electrical excitability and the shape of the action potential. Excitable cells were classified as AH cells (N=84) if the action potential showed a shoulder on the falling phase, otherwise as S cells (N=56). Cells in which no action potential could be evoked by current injection were classified as non-spiking (NS) cells (N=49). The three classes of cells showed significant differences with respect to membrane potential, input resistance and fast synaptic input. The AH cells had significantly larger somata ($p<0.01$) than the S cells. The NS cells were significantly smaller than the AH and S cells ($p<0.01$). AH and S cells were found to be randomly located in the ganglia, whereas the NS cells clustered ($P<0.008$) in close proximity to the onsets of internodal strands. We conclude that the shoulder of the action potential can be used as a single criterion to distinguish 'on line' S and AH neurons unequivocally.

Introduction

There is abundant evidence for the existence of a heterogeneous population of neurons in the enteral nervous system (reviewed by Wood, 1989). Dogiel (1899) was the first to describe three morphological types of neurons based on methylene

blue staining and proposed the shapes and functions of the cells to be related. More recently, it has been shown that the heterogeneity of the myenteric neuronal population also concerns the electrical properties of the cells (Nishi and North, 1973; Hirst *et al.*, 1974), and the presence of specific neurotransmitters and neuromodulators (Bornstein *et al.*, 1984; Costa *et al.*, 1987; Furness *et al.*, 1988; Sternini, 1988; Brookes *et al.*, 1991).

With respect to the electrical properties, three different cell types have been distinguished by means of intracellular recording. These types are S or Type 1 neurons receiving fast synaptic input, AH or Type 2 neurons showing a longlasting after-hyperpolarization (Nishi and North, 1973; Hirst *et al.*, 1974) and Type 3 cells which are inexcitable (Nishi and North, 1973). Recently, it has become evident that not only are there more electrical differences between the cell types, but also that these differences are dependent on conditions, such as the presence of neurotransmitters or neuromodulators in the ganglion (Wood, 1989). Neurotransmitters for slow synaptic excitation, for instance, may augment excitability and suppress the after-hyperpolarization in AH neurons (reviewed by North, 1982). Further, electrophysiological evidence (Grafe *et al.*, 1979; Katayama *et al.*, 1986; Iyer *et al.*, 1988) and the morphologically demonstrated presence of synapses on electrophysiologically characterized AH neurons (Erde *et al.*, 1985) show clearly that some of the AH neurons receive synaptic input. This property has for a long time been thought to be exclusive to S cells (Hirst *et al.*, 1974; Bornstein *et al.*, 1984; Surprenant, 1994). Moreover, the properties of these three cell types differ in the several parts of the gastro-intestinal tract of the guinea-pig, such as the corpus (Schemann and Wood, 1989) and antrum (Tack and Wood, 1992) of the stomach, the colon (Frieling *et al.*, 1991) and the rectum (Tamura and Wood, 1989). For these reasons, S and AH neurons cannot always unequivocally be distinguished in electrophysiological studies. Therefore it seems necessary to develop selective criteria for distinguishing the different types of neurons in the different plexus preparations.

The main difference between the S and AH neurons, with respect to electrical properties, is the calcium contribution to the inward current during an action potential in AH neurons (Tatsumi *et al.*, 1988), which is absent in S neurons (Hirst and Spence, 1973; North, 1973; Hirst *et al.*, 1974; Wood and Mayer, 1978; Hirst *et al.*, 1985a,b; Iyer *et al.*, 1984; Tamura and Wood, 1989). As a consequence, the action potentials of S neurons can be totally blocked by tetrodotoxin, whereas AH neurons have a tetrodotoxin resistant inward Ca^{2+} current during the action potential (Hirst and

Spence, 1973; North, 1973; Grafe *et al.*, 1980; Hirst *et al.*, 1985a, Tamura and Wood, 1989). This calcium contribution is visible in AH neurons as an inflexion on the falling phase of the action potential (further referred to as the shoulder (see Fig. 3)). This inflexion can be prolonged by tetraethylammonium or depletion of external calcium (Wood *et al.*, 1979; Hirst *et al.*, 1985a; Tamura and Wood, 1989). Thus, one would expect that it would be possible to use the shoulder as a single and selective criterion to distinguish S from AH neurons.

The morphology of myenteric neurons has been shown to be correlated with their electrophysiological properties. In general, AH neurons are reported to have Dogiel type II morphology, i.e. they have large, smooth cell somata with many long processes (Erde *et al.*, 1985; Katayama *et al.*, 1986; Iyer *et al.*, 1988; Bornstein *et al.*, 1991). A great majority of S neurons have Dogiel type I morphology, i.e. a single long process and a number of short processes (Bornstein *et al.*, 1984; Katayama *et al.*, 1986). Not much quantitative information is available, however, about the soma size of the three electrophysiologically different cell types. Further, nothing is known about the location of electrophysiologically different classes of cells within a ganglion. Such information might give insight into the functional arrangement of neurons within the enteric nervous system.

In the present study, we have investigated whether it is possible to use the shoulder as a single criterion to distinguish S from AH neurons. Further, the correlations between cell type, soma size and location within the ganglion were determined from unstained preparations. The results show a significant difference in passive electrical properties, fast synaptic input and soma size between the three cell types. The location of the excitable cells in a ganglion was not related to the other cell properties. A preliminary account of these findings has been published in abstract form (Schutte *et al.*, 1992a)

Material and methods

Preparation

Guinea-pigs (200 - 300 g) were stunned by a blow to the head, decapitated and exsanguinated. The small intestine was removed and a 2 cm segment of the distal ileum was slipped onto a glass rod. A small cut was made along the mesenteric border and both muscle layers with the myenteric plexus were stripped away with blunt forceps. This preparation was then placed in a dissection dish and perfused with

ice-cold Krebs solution. It was pinned flat under stretch to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI) at the bottom of the dish. The circular muscle layer was removed with fine forceps to expose the myenteric plexus on the longitudinal muscle layer. The preparation was then transferred and pinned to Sylgard resin at the glass bottom of a 1.5 ml recording chamber (diameter 21 mm). The chamber was perfused at a rate of 3 ml/min with Krebs buffer, which had the following composition (mM): NaCl 120; KCl 6.0; $MgCl_2$ 1.2; NaH_2PO_4 1.4; $NaHCO_3$ 14.4; $CaCl_2$ 2.5; glucose 12.7; gassed with 95% O_2 - 5 % CO_2 and maintained at 36 °C and pH 7.4. Nifedipine (1-2 μM) was added to the perfusion fluid to prevent mechanical activity of the muscle layer.

Electrophysiological recording

The tissue chamber was placed on the fixed stage of a Zeiss UEM microscope equipped with a Hoffman Modulation Contrast system (Hoffman, 1977). Individual cells in a ganglion were visualized by a 30x objective (total magnification x 600) with a working distance of 10 mm. The ganglion from which recordings were made was immobilized using L-shaped stainless steel wires pressing on the preparation (Wood and Mayer, 1978).

Intracellular recordings were made using glass electrodes made of borosilicate glass (WPI; 1BI00F) on a Brown-Flaming micropipette puller (Sutter Instruments Co.; P-87). The electrodes were bent (Hudspeth and Corey, 1978) about 90 degrees at about 6 mm from their tips (Fig. 1), filled with 3 M KCl and had resistances of 60-80 MΩ. Under visual control selected cells were penetrated in the direction perpendicular to the surface of the preparation, by applying an oscillating voltage to the electrode tip.

Potentials were recorded with an electrometer (WPI; Intra 767), by which rectangular current pulses were also injected. Voltage and current signals were displayed on an oscilloscope. After amplification and low pass filtering (2.6 kHz) each signal was digitized using a 1401plus CED interface (Cambridge Electronic Design Ltd.) at a sampling rate of 5.6 kHz and displayed and stored on a PC.

Electrical stimulation

An etched stainless steel stimulating electrode (tip diameter 10 μm) was used to stimulate an internodal strand connected to the ganglion with extracellular current pulses of 0.15 ms duration (<0.5 Hz) produced by a stimulus isolation unit. The electrode was usually positioned at a distance of at least 200 μm away from the

impaled neuron.

Data analysis

Signals were analyzed "off line" using standard CED software (Spike2). The input resistance of the cells was measured from the voltage response to passing hyperpolarizing current pulses of constant small amplitude through the cell membrane. The durations of action potentials in impaled neurons were measured as the width of the action potential at half the height (Hirst *et al.*, 1985a); i.e. the interval between the point at which the action potential was half maximal on the rising phase and the equivalent point on the repolarization phase. The rate of decay of the action potential was measured from peak to end of action potential repolarization.

Classification of cells

Cells were classified electrophysiologically 'on line' by using 2 criteria. Firstly, cells in which an action potential could not be evoked by current injection (150 ms), regardless of the amount of evoked depolarization (until 0 mV), were classified as non-spiking (NS) cells. Secondly, cells in which the action potential evoked by current injection showed a pronounced inflexion (shoulder) on the falling phase, were classified as AH cells, the others as S cells (Fig. 3).

Morphology

Photomicrographs were taken through the microscope optics of all recorded cells. The slides were projected in a photographic enlarger (9 x) and drawings of the ganglia and corresponding cells were made. From the drawings the soma size of each cell was determined by use of a computerized image analysis system after cell circumferences had been traced by hand. The size of the long and short axis of the cells was measured from the drawings as indicated in Figure 7.

In order to compare the location of the different cell types within the ganglia, a ganglion with the shape and size of an average ganglion was constructed (Fig. 9). All drawings of ganglia were scaled to the size of the constructed ganglion and fitted on the ganglion by using the length axis and the internodal strands as a reference. All cells of each electrophysiologically defined cell type were drawn in a separate ganglion.

Statistics

All values are given as the mean ± standard error of the mean (S.E.M.). An unpaired t-test was used to test electrophysiological parameters for significance of difference in means. A Mann-Whitney U test was used to test the morphological parameters of the cells, which did not show a normal distribution. The distribution of the location of the cells throughout the ganglion was tested by the Chi-square test. For this, the ganglion was divided into three equal sized parts (see Fig. 9). Probability <0.05 was accepted as significant.

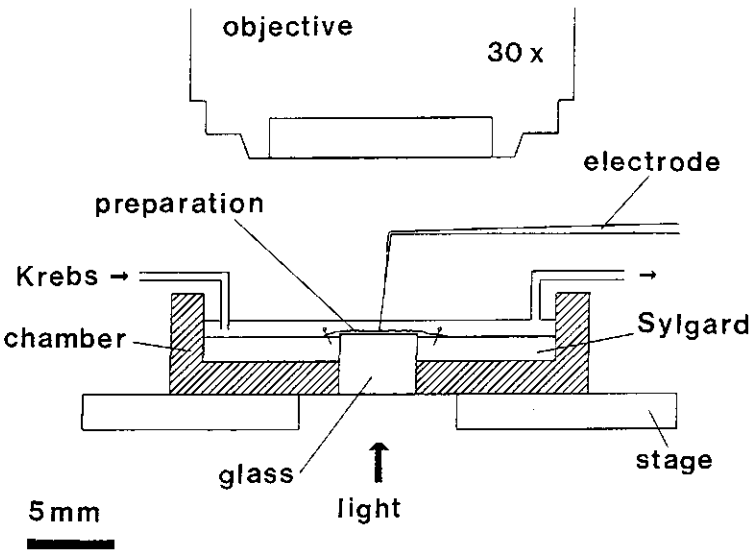

Figure 1. Schematic drawing of the tissue chamber. Intracellular recording electrodes were bent about 90 degrees at approximately 6 mm from their tips.

Results

Clear images of the cell somata of the ganglia of the myenteric plexus were obtained with the Hoffmann Modulation Contrast (HMC) system at large magnification (600 x) (Fig. 2). Due to the visual control over the cells and over the electrode, many cells (up to 8) could be studied in a single ganglion (on average 3).

The results were obtained from 189 cells in 64 ganglia. All cells had resting membrane potentials more negative than - 40 mV (-63 ± 1 mV) and were stable for at least 15 minutes. The excitable cells showed action potentials that depolarized beyond zero mV membrane potential. Recordings of cells were made for up to 3 hours.

Figure 2. Image of a ganglion of the guinea-pig myenteric plexus observed with Hoffmann Modulation Contrast optics revealing neurons within the ganglion. Two neurons are indicated by an arrow. L=longitudinal muscle layer, C=circular muscle layer. Bar = 30 μm.

Classification of cells

Three different cell types, S, AH and non-spiking (NS) cells, were distinguished. The cells were distinguished on the basis of their ability to produce action potentials. All cells that were not excitable upon intrasomal current injection, even when strongly depolarized (until 0 mV), were classified as NS cells. The electrically excitable cells showed at least one action potential at the onset of a depolarizing current pulse. Those cells that showed an inflexion on the falling phase of the action potential, (shoulder; Fig. 3) were classified as AH neurons. The cells that did not, as S neurons. Examples of the action potential shape of the two cell types

are shown in Figure 3. It is important to note that, as can be seen in Figure 3 and which was the case for all cells, the shoulder was either absent (S neurons) or so prominently present (AH neurons) that visual recognition was unequivocally possible. In this way, 84 cells were classified as AH neurons, 56 as S neurons and 49 as NS cells.

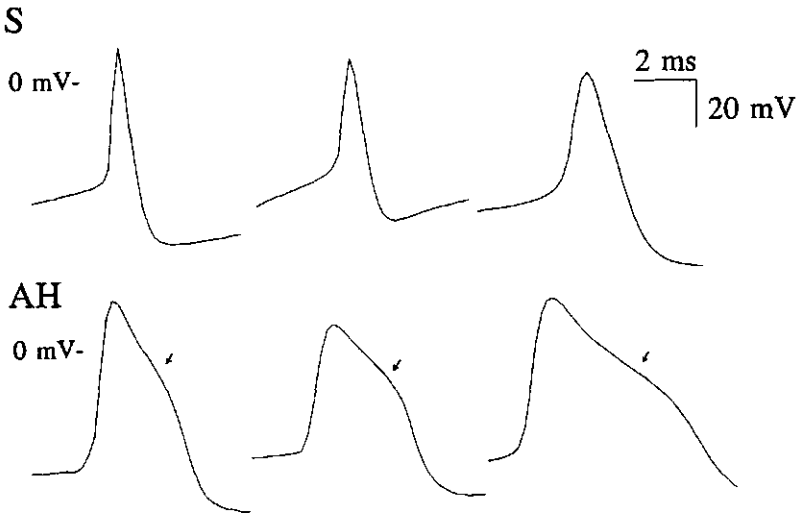

Figure 3. Difference in action potential shape of three S (**top**) and three AH (**bottom**) neurons. The action potentials of the AH neurons show a pronounced inflexion on the falling phase (shoulder) as indicated by the arrow, whereas the action potentials of the S neurons do not.

Properties of cells

AH neurons. AH neurons (N=84) had membrane potentials of -65 ± 1 mV and input impedances of 70 ± 5 MΩ. The duration of the action potential was 1.9 ± 0.04 ms (Table 1), and the amplitude was, on average, 88 ± 2 mV. The mean rate of decay of the action potential was 40 ± 2 V/s (Table 1). Of the AH neurons, 70 % (59/84) showed one action potential at the onset of the current injection. Increasing the strength of the depolarizing current pulses did not increase the number of action potentials. These cells showed a longlasting after-hyperpolarization, with durations of at least 4 sec (Fig. 4A). During the after-hyperpolarization, the input resistance of the cell was decreased, as revealed by the decreased amplitude of the voltage response to intrasomal injection of constant amplitude hyperpolarizing pulses (Fig. 4A).

Thirty percent of the AH neurons showed multiple action potentials (up to 9 in 150 ms) upon intrasomal stimulation (Fig. 4B). No longlasting (> 4 s) after-hyperpolarizations were associated with the action potentials of these cells. Properties of AH neurons giving multiple action potentials differed from those giving a single action potential in that they had larger input impedances (93 vs. 56 MΩ) and a lower threshold for spike discharge (-48 vs. -40 mV). These more excitable AH neurons also produced action potentials at the offset of a hyperpolarizing current pulse as S neurons do. After a short application of tetrodotoxin (10 nM) in the superfusion fluid, these more excitable AH neurons were found to have changed their behaviour into that of the AH neurons having one action potential followed by an after-hyperpolarization.

Table 1. Electrical properties of AH, S and NS cells.

Cell type	AH	S	NS
Number of cells	84	56	49
Passive properties			
Membrane potential (mV)	-65 ± 1^{a}	-57 ± 1^{b}	-69 ± 1^{c}
Input resistance (MΩ)	70 ± 5^{ab}	80 ± 6^{a}	39 ± 5^{b}
Action potential properties			
Duration (ms)	1.9 ± 0.04^{a}	1.0 ± 0.04^{b}	
Rate of decay (V/s)	40 ± 2^{a}	58 ± 2^{b}	
Synaptic properties			
fEPSPs (% of cells)	24^{a}	95^{b}	44^{a}
Amplitude (mV)	5 ± 1^{a}	12 ± 2^{b}	8 ± 2^{a}
Duration (ms)	20 ± 3	23 ± 2	22 ± 3
Antidromic responses (% of cells)	65^{a}	5^{b}	4^{b}

[a,b,c]) Values (means ± S.E.M.) in the same horizontal row with different letter superscripts are significantly different from each other (t-test; $p<0.05$).

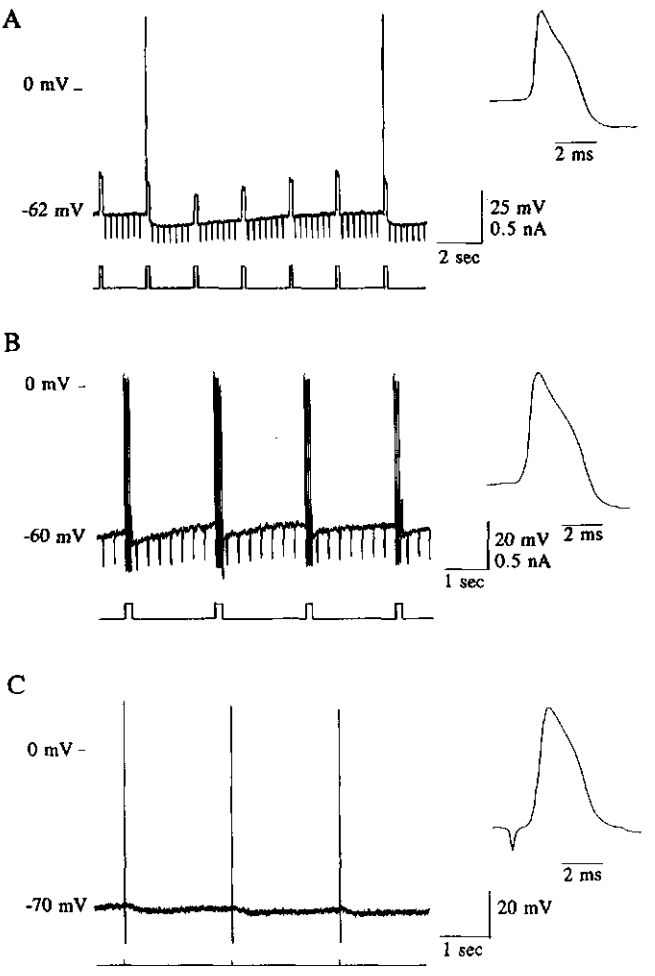

Figure 4. Intracellular recordings obtained from AH neurons of the myenteric plexus. **A:** Injection of a depolarizing current pulse (150 ms; 0.5 Hz; 0.2 nA) induces a single action potential followed by a long after-hyperpolarization (AH). Identical current pulses injected during the AH fail to elicit action potentials. Decreased input resistance during AH is reflected by decreased amplitudes of electronic potentials produced by repeated injection of hyperpolarizing current pulses. Resting membrane potential (rmp) is -62 mV. **B:** Recording of an AH neuron (rmp=-60 mV) which produces repetitive action potentials through-out the duration of a depolarizing current pulse (0.15 nA). Note the absence of the long AH. **C:** Stimulation of an internodal strand with current pulses by an extracellular electrode (0.5 Hz; 0.15 ms) evokes antidromic responses, followed by an AH. Rmp = -70 mV. **Insets** show the shapes of the action potentials.

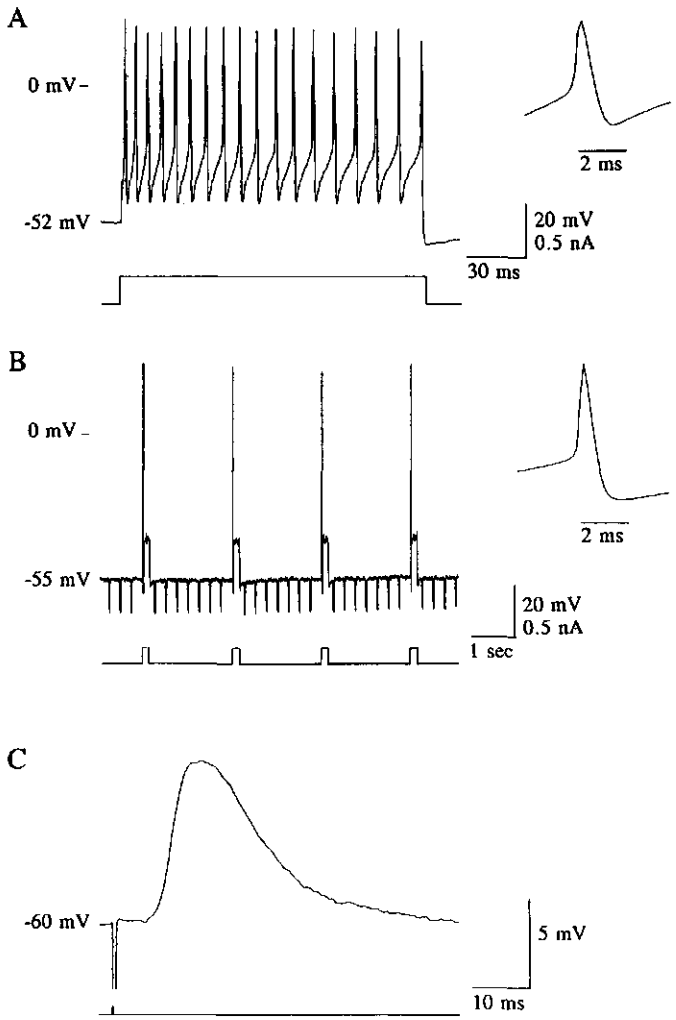

Figure 5. Intracellular recordings obtained from S neurons of the myenteric plexus. **A:** Injection of a depolarizing current pulse (150 ms; 0.5 Hz; 0.25 nA) through the recording electrode causes repetitive spiking. Resting membrane potential (rmp) is -52 mV. **B:** Example of a neuron (rmp = -55 mV) in which depolarizing current pulses (150 ms; 0.5 Hz; 0.15 nA) only evoke one action potential on each current pulse. Increasing the amount of depolarization did not increase the number of action potentials. **C:** Stimulation of an internodal strand with current pulses by an extracellular electrode (0.5 Hz; 0.15 ms) induces a fEPSP. Recording shows a computerized average of 65 fEPSPs. Rmp = - 60 mV. **Insets** show the shapes of the action potentials.

S neurons. 56 Cells were classified as S neurons, of which 66 % (37/56) showed multiple action potentials (maximum 21 in 150 ms) upon intrasomal current injection (Fig. 5A). 34 % of the S cells showed one single action potential at the onset of the current pulse (Fig. 5B). S neurons had a lower resting membrane potential (-57 ± 1 mV) and a higher input impedance (80 ± 6 MΩ) than the AH neurons (Table 1). The frequency distribution of the duration of the action potentials of the, by the shoulder identified, AH and S cells is given in Figure 6. It appears that these two cell populations can also be distinguished by the action potential duration, which was measured as the width of the action potential at half the height (Hirst *et al.*, 1985). The mean duration of the action potential was shorter in S cells (1.0 ± 0.04 ms; Table 1) than in AH cells (1.9 ± 0.04 ms). The mean rate of decay of the action potential was significantly ($P<0.001$) larger in S neurons (58 ± 2 V/s) than in AH neurons. The amplitudes of the action potentials in S neurons were found to be smaller than those in AH neurons (74 vs. 88 mV). This is in accordance with the difference in membrane potential between the cell types.

S neurons that showed only one action potential differed from the others in having lower input impedances (61 vs 91 MΩ) and a higher threshold value for spike discharge (-35 vs -43 mV).

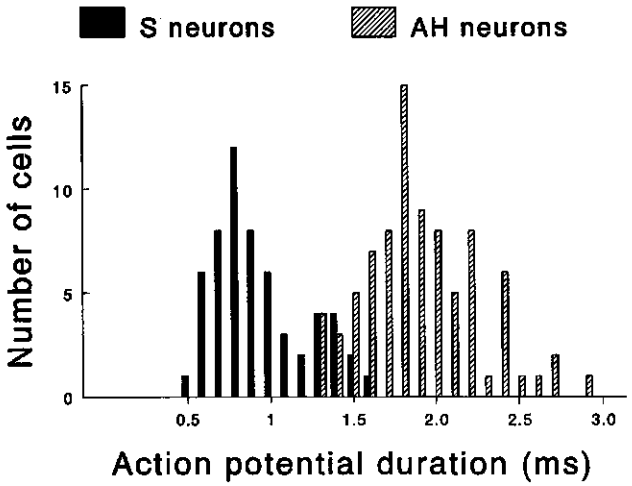

Figure 6. Histograms of the frequency distribution of the duration of the action potentials of the populations of AH and S neurons. The total number of AH and S cells was 84 and 56, respectively.

Non-spiking (NS) cells. Sometimes cells which were initially found to be inexcitable became excitable within the first half hour of impalement. Only cells that were not electrically excitable after 45 minutes of stable impalements were classified as NS cells. On penetration of a NS cell a steady resting membrane potential of on average -69 ± 1 mV was recorded and the average input impedance of these cells was lower (39 ± 5 MΩ) than that of S and AH cells.

Synaptic properties of the cell types

By electrical stimulation of an internodal strand connected to the ganglion, antidromic responses and fast and slow synaptic responses could be evoked. Antidromic responses were distinguished from fast EPSPs (fEPSPs) by their shorter duration (< 5 ms) and by retention of a constant amplitude when the membrane potential was current clamped to hyper- or depolarized levels relative to the resting potential (cf. Wood, 1989). The rise time for the fEPSPs was on average 4.6 ± 0.1 ms, in contrast to about 1 ms for the antidromic responses. The stimulation electrode was placed at one specific location for each impaled cell, so that only qualitative information on the cell's input was obtained.

Almost all S cells (41/43) showed fEPSPs upon stimulation of an internodal strand (amplitude 12 ± 2 mV; duration 23 ± 2 ms; Table 1). Figure 5C shows an example of an averaged fEPSP. Of the impaled AH neurons, 24 % (14/59) responded to electrical stimulation with a fEPSP (amplitude 5 ± 1 mV; duration 20 ± 3 ms; Table 1). About half of the NS cells (11/25) were found to respond to electrical stimulation of an internodal strand with a fEPSP (amplitude 8 ± 2 mV; duration 22 ± 3 ms; Table 1). The fEPSPs in the NS cells never resulted in discharge of action potentials, whereas in AH and S cells they often did.

More than half of the AH neurons (39/60) showed antidromic responses upon stimulation of an internodal strand, followed by an after-hyperpolarization. The shoulder was clearly visible on the action potentials that invaded the cell soma antidromically (Fig. 4C). Only 2 out of 43 S cells showed antidromic responses.

Size and shape of cells

The somata of the cells were clearly visible (Fig. 2) through the microscope. The cells showed a wide variation in shape, which generally varied from round to oval. For most cells the long axes of the soma lay in the same direction as the length axes of the ganglion (Fig. 7). During the course of an impalement some cells filled out and seemed to increase in size. This swelling was not associated with significant

changes in electrical and synaptic properties of the cells (cf. Gabella and North, 1974).

Photomicrographs were taken before and after impalements of 141 electrophysiologically identified cells and drawings of the cell somata in the ganglia were made (see Methods). The soma sizes expressed as surface area, showed a wide variation, the smallest cell being 130 μm^2 and largest being 2600 μm^2.

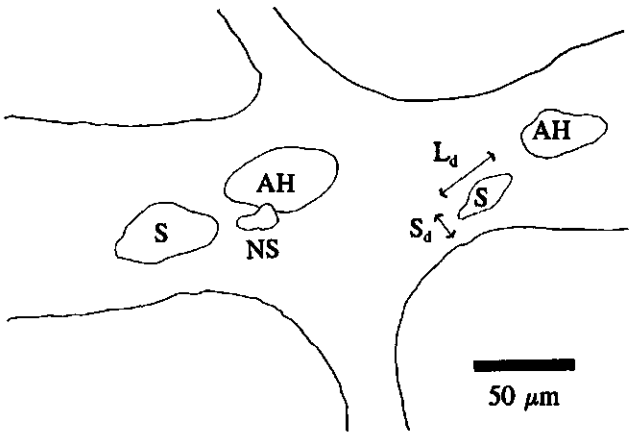

Figure 7. Example of a ganglion containing five electrophysiologically identified cells made from a photomicrograph of an unstained preparation of the guinea-pig myenteric plexus. Note the differences in size and shape of the somata between the cell types (indicated by AH, S and NS) and also between the cells belonging to one type. How the long (L_d)and short axis (S_d) of the somata were measured is indicated.

Figure 7 shows a drawing of a ganglion containing five electrophysiologically classified cells. How the size of the long and short axis of cells were measured is indicated. The correlations between long axis diameter and short axis diameter for the different cell types are given in Figure 8A. AH cells generally had large ovoidal appearances with the long axis almost twice the size of the short axis (Fig. 8A; Table 2; cf. Bornstein *et al.*, 1991). The mean soma size of AH cells was 1290 ± 84 μm^2 (Table 2). The S cells were smaller than AH cells, having a mean surface area of 760 ± 65 μm^2 ($p<0.01$). S cells showed the largest variation in shape, being round, oval or sometimes very complicated in appearance. The NS cells appeared to be small, round cells, but a few larger ovoidal appearances were also found. Their mean soma size was smaller (430 ± 57 μm^2; $p<0.01$) than that of the excitable cells (Table 2).

Table 2. Soma sizes of the three cell types.

Cell type	AH	S	NS
Number of cells	66	47	34
Long axis (μm)	53 ± 3[a]	39 ± 2[b]	27 ± 2[c]
Short axis (μm)	30 ± 1[a]	23 ± 7[b]	18 ± 1[c]
Surface area (μm^2)	1290 ± 84[a]	760 ± 65[b]	430 ± 57[c]

[a,b,c]) Values (means ± S.E.M.) in the same horizontal row with different letter superscripts are significantly different from each other (Mann-Whitney U test; $p<0.01$).

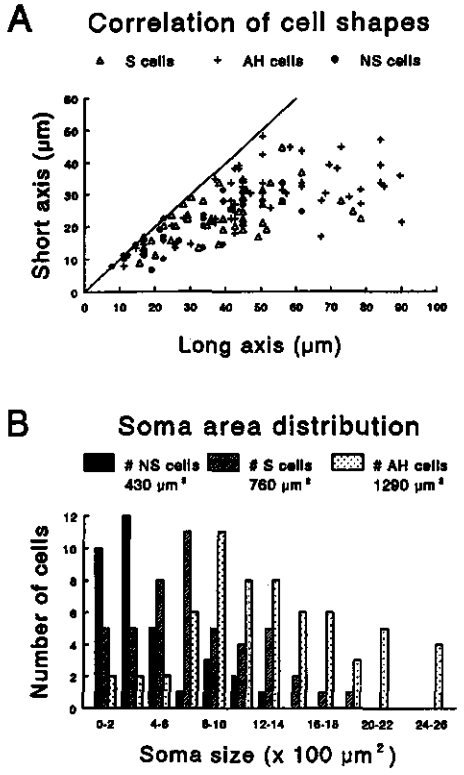

Figure 8. **A:** The cell shape distribution obtained by plotting the short axis diameter against the long axis diameter for the three different cell types. The line represents cells with round somata for reference. **B:** Histograms of the frequency distributions of the measured soma sizes of the populations of the three electrophysiologically identified cell types. Note that, although the means are significantly different (Mann-Whitney U test; $p<0.01$), there is an overlap in soma sizes for the three cell types. The total number of AH, S and NS cells was 66, 47 and 34, respectively.

Although mean soma sizes were significantly different for the three cell types, an overlap in size occurred for the different populations of cell types (Fig. 8B). No correlation was found between the presence of fast synaptic input on the NS cells and the size of these cells. However, all NS cells with a soma size above 800 μm^2 (7 cells; Fig. 8B) showed some sign of neural properties, such as, for instance, spiking upon impalement, fast synaptic input or antidromic responses.

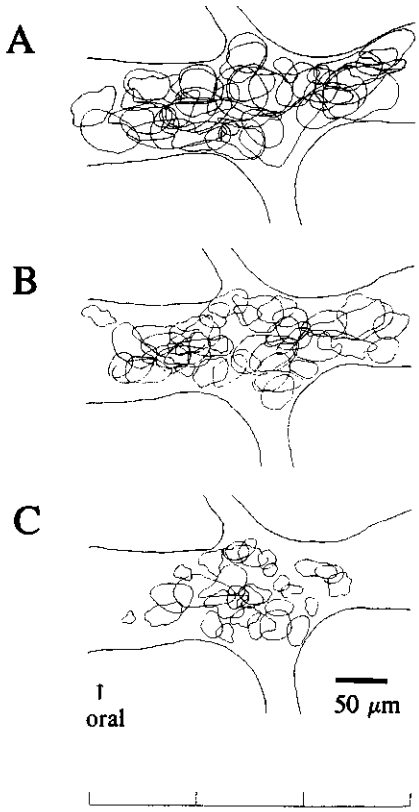

Figure 9. Location of the three different cell types within the ganglion that was constructed using the length axis of the ganglia and the internodal strands as a reference (see Methods). All cells belonging to a specific cell type (AH, S and NS) are depicted in a ganglion. The bar indicates the three equal-sized parts in which the ganglion was divided for statistical testing of cell distribution. **A:** The AH cells can be seen to have large cell somata and to be randomly located throughout the ganglion. **B:** The S cells are smaller in size than the AH cells and are also located randomly throughout the ganglion. **C:** The NS cells are smaller in size than the AH and S cells and are located in close proximity to the onsets of the internodal strands (Chi-square test; $p<0.008$).

Location of cells

In order to compare the locations of cells within the ganglia (which were found to have roughly the same size) a schematic ganglion was drawn with two internodal strands (see Methods). All cells of one particular identified type were then drawn in the ganglion with regard to their position to the strands, to the length axis of the ganglion and to the oral/aboral direction (Fig. 9).

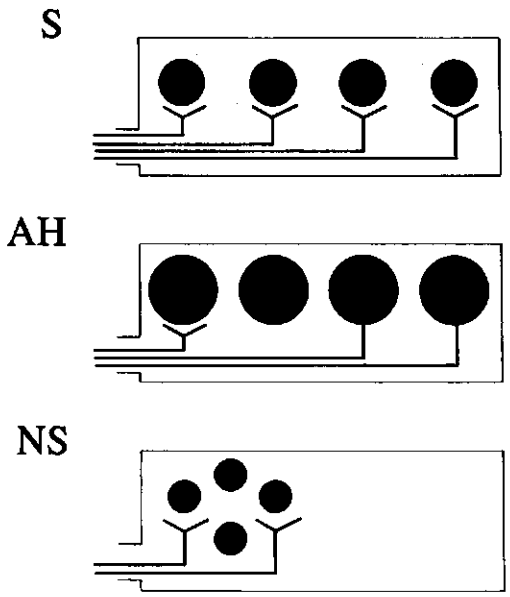

Figure 10. Highly schematized summary of the results of measurements of electrophysiological and morphological properties of the three cell types. Four cells are depicted as circles for each cell type in a separate schematic ganglion with one internodal strand. The equal number of cells per ganglion indicates that the number of cells encountered was about the same for each cell type. The differences in diameter of the circles reflect the measured differences in mean soma size. The measured frequency of occurrence of fast synaptic input on each cell type is indicated by the percentage of the four cells for which synaptic connections are drawn. The measured frequency of occurrence of antidromic responses of the cells to electrical stimulation of the internodal strands, which is assumed to give an indication of the amount of processes of the soma, is indicated by the percentage of the four cells that are connected directly to the internodal strand.

It appeared that the S and AH cells were randomly located through the ganglion. Also no special relationship was noticed in the positions of the different cell types with regard to each other. The NS cells, especially the smallest ones, seemed to cluster in the proximity of the internodal strands (Fig. 9). A Chi-square Test on all NS cells showed that the number of cells in close proximity to the internodal strands (for details see Fig. 9) was indeed significantly ($p<0.008$) larger than that in the other parts of the ganglion.

Summary of results

Figure 10 depicts a schematic summary of the above results with respect to the electrophysiological measurements of the connections of the cells and the morphological measurements of soma sizes for the three cell types. From the figure it can be seen that the AH cells have on average the largest soma size, send many processes into the internodal strands and receive relatively little fast synaptic input. The S cells are smaller in size than the AH cells, send only a limited number of processes into the internodal strands and receive abundant fast synaptic input. The NS cells are relatively small cells, which do not seem to have many processes and of which half of the cells receive fast synaptic input.

Discussion

Classification of cells

The present results show that the neurons of the myenteric plexus of the guinea-pig ileum can be electrophysiologically classified in a consistent way in three different types by using two criteria. These criteria are the ability of the cells to produce action potentials, and if so, the occurrence of a shoulder on the falling phase of an action potential.

The electrical properties of the three classes of cells distinguished in this way are in accordance with the differences in electrical properties between three populations of cells described previously (reviewed by Wood, 1989). For instance, our finding that AH neurons have more negative membrane potentials and lower input resistances than S neurons (Table 1) is in good agreement with previous data (Nishi and North, 1973; Hirst *et al.*, 1974; Wood, 1989). Also, the observation that the non-spiking cells have the lowest values for these parameters confirms existing data (Nishi and North, 1973; Wood, 1989).

The selection criterion used in our classification of the excitable cells is based on the fact that action potentials in AH neurons always show an inflexion on the falling phase. These inflexions are also apparent in previously published work (Hirst *et al.*, 1974; Wood and Mayer, 1978) and no such shoulder occurs on the repolarization phase of the action potentials in S neurons (Iyer *et al.*, 1984). The shoulder has shown to be prolonged by tetraethylammonium or depletion of external calcium (Wood *et al.*, 1979; Hirst *et al.*, 1985a) and to be tetrodotoxin-resistant (Hirst and Spence, 1973; North, 1973). The information available suggests that in AH neurons the inward currents during the rising phase of the action potential are carried by Na^+ and Ca^{2+} ions (Tatsumi *et al.*, 1988), the Ca^{2+} current being the cause of the inflexion (Hirst and Spence, 1973; Hirst *et al.*, 1985a). The finding that action potentials of the AH and S cells had significant different durations (cf. Iyer *et al.*, 1988), with almost no overlap in the frequency distribution (see Fig. 6), puts the classification on a firm quantitative basis.

The name AH cell does refer to the after-hyperpolarization which follows the action potential (Hirst *et al.*, 1974). An after-hyperpolarization was seen in 70% of the AH cells, the other 30% of the AH cells were in a state of augmented excitability which resembles the behaviour of S neurons. In this excitable state the hyperpolarizing afterpotentials are presumably suppressed by the action of neurotransmitters for slow synaptic excitation (Wood, 1989). The shoulder on the action potential is, however, always present. The fact that tetrodotoxin changes the electrical behaviour of the highly excitable cells into that of the other AH cells supports the notion that all cells with a shoulder belong to one class of cells.

Originally, the name S cell was used to signify the always present fast synaptic input, whereas AH cells supposedly did not receive such input (Hirst *et al.*, 1974; Bornstein *et al.*, 1984). According to the present classification, all S neurons and 24 % of the AH neurons were found to receive fast synaptic input. Our data support the notion, based on electrophysiological evidence (Grafe *et al.*, 1979; Katayama *et al.*, 1986; Iyer *et al.*, 1988) that some AH neurons do receive fast synaptic input. Morphological evidence for the presence of synapses on electrophysiologically identified AH neurons has been presented by Erde *et al.* (1985). The extent of coverage by morphologically identifiable synaptic contacts did not differ between AH and S cells. Besides fast synaptic input, slow synaptic potentials can be recorded in AH cells in response to repetitive stimulation of an interganglionic fibre tract (Erde *et al.*, 1985; Wood, 1989). Thus, synaptic input cannot be used to distinguish between S and AH neurons (cf. Erde *et al.*, 1985).

Of the S neurons, 34 % were found not to discharge action potentials continuously throughout a depolarizing current pulse, but only at the onset. According to Wood's criteria (1989) they would have been classified as AH neurons. These S neurons have been shown to have a morphology similar to the other S neurons, in that they also have a single long process (Bornstein *et al.*, 1991; Tamura, 1992).

Some of the NS cells found in this study may have been glial cells. Surely, the NS cells receiving fast synaptic input and those that showed signs of neuronal properties were neurons. This is in accordance with the morphological demonstration by Erde *et al.* (1985), that some electrophysiologically identified NS cells are neurons. Further, some of the NS cells are found to become excitable upon administration of slow excitatory modulators (Frieling *et al.*, 1993; Schutte *et al.*, 1992b). This supports the hypothesis that part of the silent cells are cells which are constituents of neural circuits that are only active during some phases of intestinal function and inactive during additional phases (Wood, 1989).

We conclude that the presence of a shoulder on the action potential is a reliable criterion for distinguishing S and AH neurons. The advantages of the present classification are that it can be used 'on line' and that it is highly selective as well as consistent, being based on the presence of a calcium contribution to the inward action potential current. This calcium contribution is not affected by the presence of neurotransmitters or neuromodulators in the ganglion. The classification of excitable enteric cells in two classes does of course not implicate that only two different cell types are present in the myenteric plexus. Based on for instance histochemical properties, morphology and responses to neurotransmitters many subclasses can be distinguished (Costa *et al.*, 1987; Furness *et al.*, 1990).

Morphology

A second purpose of this study was to correlate cell soma size with electrophysiological identification of enteric neurons. The morphology of electrophysiologically identified neurons of the myenteric plexus has been described previously by means of intracellular dye injection (Bornstein *et al.*, 1984; Hodgkiss and Lees, 1983; Katayama *et al.*, 1986). In the present study, the morphology was studied using an optical technique (HMC), which reveals no details on cell morphology. The use of this technique, however, enabled us to report differences in soma size and shape between the three identified cell populations, as seen prior to visually controlled impalement with a microelectrode. That impalement of myenteric neurons may influence their later appearance has been shown by Gabella and North

(1974) using electronmicroscopy, who reported impaled cells to increase up to 2-4 times in size during the course of impalements.

The observation that AH neurons are larger than S neurons is in accordance with data obtained by correlating electrophysiological properties of neurons with their size by using intracellular dye injection (Bornstein *et al.*, 1984; Hodgkiss and Lees, 1983) and supports the usefulness of the shoulder criterion.

Although our morphological data do not provide information on the exact number of the soma processes, some information about the presence of processes was obtained from the cell responses to electrical stimulation of the internodal strands. The high occurrence of antidromic responses in AH neurons (65%), for instance, indicates that these neurons send many processes into the internodal strands. This is in accordance with the demonstration by intracellular injection of the marker substance biocytin that AH neurons project circumferentially to nearby ganglia (Bornstein *et al.*, 1991) and supports the classification by the shoulder criterion.

Most likely, the relative number of AH, S and NS cells encountered in this study (84, 56 and 49 cells, respectively) does not reflect the proportion of each cell type present in the ganglia. Since, in general, the visibility of the cells through the microscope and the duration of stable recordings increase with cell size, in our experiments the sampling of cells probably has not been aselective, despite attempts to ensure this. In other studies of neurons of the enteric nervous system, where penetration is usually done without visualization of the cells, there is also a tendency to select larger cells (cf. Bornstein *et al.*, 1984). The overlap in soma size between the electrophysiologically different cell types (Fig. 8B) prevents determination of cell type by size only.

Location of cells

In the present experiments the inexcitable NS cells were found to cluster in the proximity of the onsets of the internodal strands. Some of these NS cells, namely the smallest ones, may have been glial cells. This view is supported by the observation in the myenteric plexus of the guinea-pig small intestine of a similar clustering in proximity to the internodal strands of small cells that are immunoreactive to the glial cell marker S-100 protein (Bornstein, personal communication).

As far as we are aware, no information is available on the location of electrophysiologically different types of neurons within the ganglia of the enteric nervous system. Our demonstration that the excitable AH and S neurons are randomly located within a ganglion suggests that there is no specific order in the location of these cells.

This, however, should not be taken as evidence that there is no organization at all with respect to location within a ganglion, since for several other types of autonomic ganglia there is evidence that cells with specific neurochemical properties and projections to the same target organ lie close together (reviewed by Elfvin *et al.*, 1993).

In summary, in the myenteric plexus of the guinea-pig ileum, three electrophysiological cell types (AH, S and NS cells) can be distinguished on the basis of their electrical excitability and only one other criterion, i.e. the presence of a shoulder on the action potential. The results further show that there is a strong correlation between cell type and soma size and that the small NS cells, presumably glial cells, are located in a cluster close to the internodal strands.

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Chapter 3

CCK_A and CCK_B receptor subtypes both mediate the effects of CCK-8 on myenteric neurons in the guinea-pig ileum

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CCK_A and CCK_B receptor subtypes both mediate the effects of CCK-8 on myenteric neurons in the guinea-pig ileum

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Abstract

The effects of cholecystokinin (CCK-8) on myenteric S neurons were investigated by intracellular recording techniques, with the aim to determine the CCK receptor subtypes involved. CCK-8 (1-1000 nM) evoked concentration dependent longlasting excitatory responses in 45 of 54 neurons. CCK receptor antagonists were applied to 15 neurons in which CCK-8 evoked an excitatory response. In 5 of these neurons, application of the CCK_A antagonist L-364,718 (100-500 nM) antagonized the action of CCK-8 and the CCK_B antagonist L-365,260 (500 nM) had no effect. L-365,260 (100-500 nM) antagonized the CCK-8 induced response in 5 neurons, on which L-364,718 had no effect. In the other 5 neurons each antagonist (500 nM) partly inhibited the CCK-8 evoked excitation and application of both antagonists (500 nM) caused a complete blockade of the response to CCK-8. The selective CCK_B receptor agonist CCK-8NS had similar excitatory effects as CCK-8, but only on the neurons in which CCK-8 evoked effects were antagonized by L-365,260. The results demonstrate that the excitatory effects of CCK-8 are mediated by both CCK_A and CCK_B receptor subtypes. Further, the results indicate that some neurons possess exclusively the CCK_A or the CCK_B receptor subtype, but others possess both subtypes.

Introduction

The peptide cholecystokinin (CCK) functions in the gastrointestinal tract as a hormone involved in the control of pancreatic secretion, gallbladder contraction and gut motility (Walsh, 1994). CCK is produced by endocrine mucosal cells in the duodenum and proximal jejunum and released into the bloodstream in response to a meal (Williams, 1982). Although multiple forms of CCK are present in the gastrointestinal tract, the biologically most active form of CCK is the sulfated C-terminal octapeptide CCK-8 (Walsh, 1994).

The effects of CCK on the motility of the gastrointestinal tract are brought about either directly on smooth muscle cells or are mediated by neurons in the enteric nervous system (reviewed by Daniel *et al.*, 1989). In the small intestine of the guinea-pig, CCK causes contraction of longitudinal muscles by an indirect action on myenteric inter- or motorneurons, presumably S type neurons (Bornstein *et al.*, 1994), which release acetylcholine (Vizi *et al.*, 1973) and substance P (Lucaites *et al.*, 1991). Intracellular recordings have shown that CCK evokes mainly excitatory slow responses in myenteric S neurons of the guinea pig ileum (Nemeth *et al.*, 1985).

Two CCK receptor subtypes have been identified, namely the CCK_A (alimentary) and the CCK_B (brain) receptor (Dourish and Hill, 1987; Wank, 1995). The CCK_A receptors have a high affinity for CCK-8 and the CCK_A specific antagonist L-364,718 (Chang and Lotti, 1986; Wank, 1995) and a relatively low affinity for the nonsulfated form of CCK (CCK-8NS), CCK-4 and gastrin (Huang *et al.*, 1989; Wank, 1995). CCK_B receptors have high and nearly equal affinities for CCK-8, CCK-8NS, CCK-4 and gastrin (Huang *et al.*, 1989; Wank, 1995) and for the specific CCK_B antagonist L-365,260 (Lotti and Chang, 1989). In the CNS, the principal receptor for the neurotransmitter CCK is the CCK_B receptor, but evidence has been provided for the existence of neuronal CCK_A receptors (Boden and Woodruff, 1994).

There are some indications that apart from CCK_A (Chang and Lotti, 1986; Zelles *et al.*, 1990) also CCK_B receptors may be involved in ileum contractions evoked by CCK. Also, the results of an *in vivo* study (Rodriguez-Membrilla *et al.*, 1995) suggest that CCK-mediated motor changes after a meal in rat are due to stimulation of peripheral CCK_B receptors. The results of *in vitro* studies showed that CCK-4 and gastrin (CCK_B agonists) contract the ileum through activation of neuronal CCK_B receptors (Dal Forno *et al.*, 1992; Gaudreau *et al.*, 1987; Lucaites *et al.*, 1991; Patel and Spraggs, 1992). This suggests that CCK_B receptors may be present in the enteric nervous system.

An elegant intracellular microelectrode study has revealed that in the gallbladder the main neuronal action of CCK-8 is a presynaptic facilitatory effect on fast synaptic transmission, mediated exclusively by presynaptic CCK_A receptors (Mawe, 1991)). Intracellular studies on stomach antral (Tack *et al.*, 1992) and duodenal myenteric neurons (Mutabagani *et al.*, 1993) have suggested that CCK-8 evoked effects in these neurons are mediated solely by CCK_A receptors. Thus, although there is no doubt about the presence of CCK_A receptors on myenteric neurons, conclusive evidence about the presence of CCK_B receptors on myenteric neurons is lacking.

In the present study intracellular recordings were used to examine the effects of CCK-8 on S neurons of the myenteric plexus of the guinea pig ileum. The selective antagonists, L-364,718 and L-365,260 were used to characterize the receptor subtype involved. These antagonists are highly selective for respectively the CCK_A and the CCK_B receptor subtype and there is evidence that these antagonists do not interfere with other receptors (Chang and Lotti, 1986; Lotti and Chang, 1989; Wettstein *et al.*, 1994).

The results show that the excitatory effects of CCK-8 on S neurons are mediated by CCK_A as well as by CCK_B receptor subtypes.

Material and methods

Preparation

Guinea-pigs (200 - 300 g) were stunned by a blow to the head, decapitated and exsanguinated. All procedures were approved by the Wageningen University Committee for Experiments on Animals and were in accordance with the Dutch Law on experimental animals (1987). The small intestine was removed and a 2 cm segment of the distal ileum was slipped onto a glass rod. A small cut was made along the mesenteric border and both muscle layers with the myenteric plexus were stripped away with blunt forceps. This preparation was then placed in a dissection dish and perfused with ice-cold Krebs solution. It was pinned flat under stretch to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI) at the bottom of the dish. The circular muscle layer was removed with fine forceps to expose the myenteric plexus on the longitudinal muscle layer. The preparation was then transferred and pinned to Sylgard resin at the glass bottom of a 1.5 ml recording chamber (diameter 21 mm). The chamber was perfused at a rate of 3 ml/min with Krebs buffer, which had the following composition (mM): NaCl 120; KCl 6.0; $MgCl_2$ 1.2; NaH_2PO_4 1.4; $NaHCO_3$ 14.4; $CaCl_2$ 2.5; glucose 12.7; gassed with 95% O_2 - 5% CO_2 and maintained at 36 °C and pH 7.4. Nifedipine (1-2 μM) was added to the perfusion fluid to prevent mechanical activity of the muscle layer. The preparations were left undisturbed in the chamber for 2 h before experiments were started, to eliminate any effects of neuroactive compounds released from the plexus during the dissection.

Electrophysiological recording

The tissue chamber was placed on the fixed stage of a Zeiss UEM microscope

equipped with a Hoffman Modulation Contrast system. Individual cells in an immobilized (Wood, 1994) ganglion were visualized by a 30x objective (total magnification x600) with a working distance of 10 mm. Intracellular recordings were made using glass electrodes made of borosilicate glass (World Precision Instruments Inc.; 1B100f) on a Brown-Flaming micropipette puller (Sutter Instruments Co.; P-87). The electrodes were bent about 90 degrees at about 6 mm from their tips, filled with 3 M KCl and had a resistance of 60-80 MΩ. Under visual control selected cells were penetrated in the direction perpendicular to the surface of the preparation, by applying an oscillating voltage to the electrode tip.

Potentials were recorded with an electrometer (World Precision Instruments Inc.; Intra 767), by which rectangular current pulses were also injected. After amplification and low pass filtering (2.6 kHz) each signal was digitized using a 1401plus CED interface (Cambridge Electronic Design Ltd.) at a sampling rate of 5.6 kHz and displayed and stored on a PC.

Electrical stimulation

An etched stainless steel stimulating electrode (tip diameter 10 μm) was used to stimulate an internodal strand connected to the ganglion with extracellular current pulses of 0.15 ms duration (<0.5 Hz) produced by a stimulus isolation unit. The electrode was positioned at a distance of at least 200 μm away from the impaled neuron.

Drugs and their administration

CCK-8 and CCK-8NS were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of CCK-8 and CCK-8NS were prepared in concentrations of 50 μM in Krebs solution containing bovine serum albumin, aprotonin and bacitracin (each at 0.1%) and stored at -20 °C. CCK-8 and CCK-8NS were applied by micro-ejection from fine-tipped pipettes (tip diameter 10 μm) with nitrogen pulses of controlled pressure and duration, or by addition to the superfusion solution in known concentrations. Application of CCK-8 and CCK-8NS by micro-ejection was at intervals exceeding 2 min. Application at shorter intervals caused the responses to decline, suggesting desensitization to occur. CCK-8 and CCK-8NS (1 nM - 1000 nM) were applied in the bath for 1-5 min. Between bath applications, the tissue was kept in drugs-free solution for at least 15 minutes to ensure reproducibility.

The selective CCK_A antagonist L-364,718 and CCK_B antagonist L-365,260, were kindly provided by Dr. V. Lotti (Merck, Sharp & Dohme, Rahway, NJ). L-364,718

and L-365,260 were dissolved in DMSO (99%) and frozen at -20^0 C. Solutions at the desired concentrations were prepared just before application to the superfusion by thawing fresh aliquots in Krebs solution. In the experiments, DMSO never exceeded 0.1% in the perfusion medium. Control experiments showed that 0.1% DMSO did not affect the electrical properties of the neurons. Antagonists (1 nM - 1000 nM) were usually applied for 10 min.

In some experiments, atropine sulphate (1 μM) and tetrodotoxin (TTX; 10 nM) were added to the superfusing solution.

Data analysis

The data were analyzed "off line" using standard CED SPIKE2 software and computer programs written in the CED SCRIPT language. The membrane resistance of the cells was measured from the voltage response to hyperpolarizing current pulses through the cell membrane, by averaging at least 10 responses. The time course of CCK-induced slow changes in membrane resistance, membrane potential and action potential frequency was determined by measuring these parameters every few (1-10) seconds. The maximum values for these parameters reached during CCK-8 superfusion were determined. Properties of fast synaptic potentials (fEPSPs) were determined by averaging at least 25 fEPSPs.

Classification of cells

Excitable cells were classified electrophysiologically "on line" by using a criterion of which we have recently shown that this distinguishes S and AH neurons unequivocally (Schutte *et al.*, 1995). Cells in which the action potential evoked by current injection showed a pronounced inflexion (shoulder) on the falling phase, were classified as AH neurons, the others as S neurons (Bornstein *et al.*, 1994; Schutte *et al.*, 1995). The classification was confirmed by "off-line" scrutinizing of the action potential shape in the digitized datafiles.

Statistics

All values are given as means ± standard error of the mean (S.E.M.). A paired t-test was used to test parameters for significance of difference in means. Probability <0.05 was accepted as significant.

responsiveness to CCK-8. Two of the 5 neurons responded upon CCK-8NS application (10-100 nM) with a concentration dependent increase in membrane resistance and action potential discharge, although no change in membrane potential occurred (Fig. 7). At a concentration of 100 nM, CCK-8NS evoked in these cells excitatory responses with an action potential discharge and resistance increase comparable to the CCK-8 (100 nM) responses of the same cell. In both neurons that responded to CCK-8NS application, the CCK-8 induced effects could be antagonized partly by L-364,718 and partly by L-365,260. The other three neurons did not show any response to CCK-8NS application.

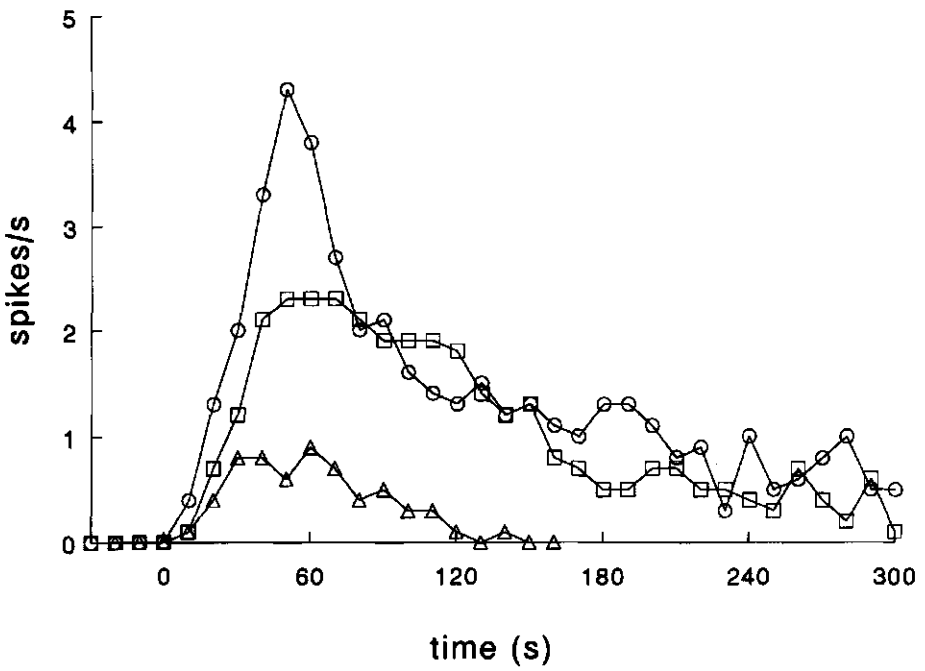

Figure 6. Time course of CCK-8 evoked change in action potential frequency. CCK-8 (100 nM) was applied from 0-90 s. On the y-axis the instantaneous action potential frequency (spikes/s; mean over 10 s) is depicted. CCK-8 application causes a longlasting burst of action potentials (o). Superfusion of L-365,260 (500 nM;□) or L-364,718 (500 nM; ▵) reduced the action potential frequency evoked by CCK-8. Superfusion of L-365,260 together with L-364,718 (each 500 nM) reduced the CCK-evoked action potential frequency to zero.

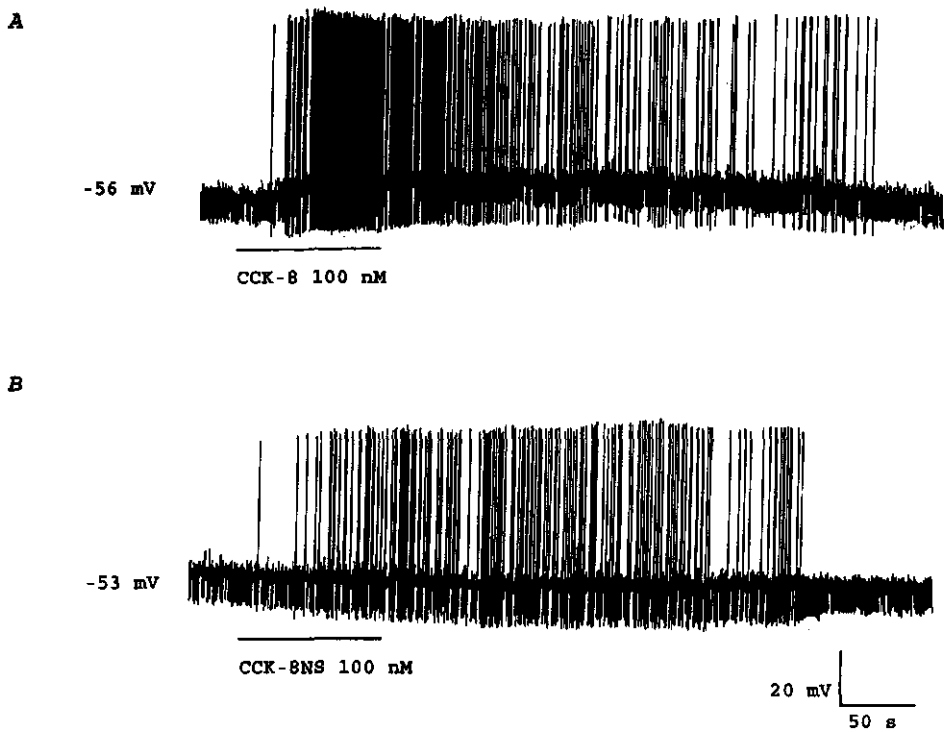

Figure 7. Comparison of the effect of CCK-8 and CCK-8NS on a S neuron. **A:** Application of CCK-8 (100 nM) evoked an increase of excitation (1.9 sp/s) accompanied by a depolarization (5 mV) and an increased membrane resistance to 115% of control. **B:** Application of CCK-8NS (100 nM) evoked an increase of excitation (0.8 sp/s) accompanied by an increase in membrane resistance to 117% of control. No change in membrane potential was detected. Application of the drugs is indicated by the bar. On the left the membrane potential is indicated.

Discussion

The application of CCK-8 caused a concentration dependent (1-1000 nM) and reversible enhancement of the excitability of most S neurons. The observed properties of the responses confirm the results of Nemeth *et al.* (1986). Evidence for the presence of CCK_A and CCK_B receptor subtypes on the neurons was obtained from observations of selective inhibition of the CCK-8 effects by antagonists and from the action of the CCK_B receptor agonist CCK-8NS.

Effects of CCK-8 on S neurons

CCK-8 caused an enhancement of the excitability in 83% of the S neurons. The results show that CCK-8 acted directly on the recorded neurons and has no presynaptic effects. Thus, in the ileum the action of CCK-8 on the neurons is different from that in the gallbladder (Mawe, 1991), where CCK-8 has exclusively a presynaptic facilitating effect on synaptic transmission.

The nanomolar concentration range at which the effects of CCK-8 were observed, is comparable to that reported for enteric neurons in the gallbladder (Mawe, 1991), for sympathetic neurons (Mo and Dun, 1986; Schumann and Kreulen, 1986) and for neurons in the CNS (Boden and Woodruff, 1994). The characteristics of the CCK-induced enhancement of excitability in S neurons are similar to the effects of other peptides, such as Substance P and Calcitonin gene-related peptide on myenteric neurons (reviewed by Wood, 1994). This excitation resembles the slow synaptic excitation that can be evoked by electrical stimulation in myenteric neurons. The ionic mechanism of this response appears to be the closure of K^+ channels (Surprenant, 1994; Wood, 1994). The finding that the CCK-8 induced responses in the neurons were accompanied by an increase in membrane resistance is also indicative of suppression of a K^+ conductance by CCK-8.

A function of CCK as neurotransmitter in the enteric nervous system has been suggested (Furness and Costa, 1989; Hollestein *et al.*, 1995; Karaus and Niederau, 1995; Walsh, 1994; Williams, 1982). Because of the lack of information on the putative neurotransmitter action of CCK in the myenteric plexus, it cannot be decided if the CCK-induced excitation observed in the present experiments resembles this action, or the endocrine action.

CCK_A and CCK_B receptor subtypes

The inhibitory effects of the selective CCK_A and CCK_B receptor antagonists on the excitatory CCK-8 responses show the presence of CCK_A as well as CCK_B receptors on S neurons in the ileum. The CCK-8 response was antagonized by L-364,718 and L-365,260 in the nanomolar (100-500 nM) concentration range. This is in accordance with the effective concentrations of the antagonists reported for other in vitro preparations (Boden and Woodruff, 1994; Chang and Lotti, 1986; Lotti and Chang, 1989). No agonist activity was observed. The finding that in some neurons the response to CCK-8 could be blocked completely by the CCK_A receptor antagonist L-364,718 was not unexpected (Chang and Lotti, 1986; Mawe, 1991; Mutabagani *et al.*, 1993; Tack *et al.*, 1992; Zelles *et al.*, 1990) and confirms the presence of CCK_A

receptors in the enteric nervous system.

Some neurons, however, were encountered in which the response to CCK-8 could be blocked completely by the CCK_B receptor antagonist L-365,260. This indicates the presence of S neurons with only CCK_B receptor subtypes. Also, neurons were found in which the CCK-8 response could be inhibited partly by the CCK_B receptor antagonist and partly by the CCK_A antagonist. Excitatory effects of CCK-8NS, a selective agonist for the CCK_B receptor (Huang *et al.*, 1989), were observed only in these neurons, and not in neurons where the CCK-8 response could be blocked completely by the CCK_A receptor antagonist. It was thus concluded that some S neurons possess both CCK_A and CCK_B receptor subtypes. The occurrence of both receptor subtypes on one neuron, as observed by us in the myenteric plexus, has also been reported for several brain regions (Boden and Woodruff, 1994).

The presence of CCK_B receptors on guinea pig stomach muscle (Bishop *et al.*, 1995) and in the small intestine (Dal Forno *et al.*, 1992; Lucaites *et al.*, 1991; Patel and Spraggs, 1992) has been suggested previously, based on results from contraction studies. Both myenteric CCK_A and CCK_B receptors are reported to be involved in the regulation of ion transport in the mouse ileum (Rao *et al.*, 1994). Recently, the presence of a high density of CCK_B receptors in the canine duodenum myenteric plexus was shown by autoradiographic analysis (Mantyh *et al.*, 1994).

The results also show that in S neurons, presumably motor- or interneurons (Bornstein *et al.*, 1994), the excitatory responses to CCK-8 mediated by the two receptor subtypes were different. Only in neurons in which CCK-8 acted through the CCK_A receptor subtype, abundant spontaneous action potential activity was observed upon application of CCK-8. Also, the duration of the response mediated by the CCK_A receptor was observed to be longer than that mediated by the CCK_B receptor. This might explain the observations from contraction studies (Dal Forno *et al.*, 1992; Lucaites *et al.*, 1991) that CCK acting on CCK_B receptors evokes a short contraction, while contractions mediated by CCK_A receptors are sustained. Further study is necessary to determine if different sub-cellular mechanisms, which may involve cAMP (Wood, 1994) or other second messengers, underlie the CCK-8 induced effects on the enteric neurons.

In conclusion, the present findings show that the excitatory action of CCK-8 on myenteric S neurons is mediated not only by CCK_A but also by CCK_B receptor subtypes. The CCK antagonists are of putative interest for the treatment of functional bowel disorders, such as functional dyspepsia and irritable bowel syndrome (Enck *et al.*, 1994; Raybould *et al.*, 1994). In fact, some of these antagonists are presently

being examined in clinical trials for their therapeutic usefulness in digestive as well as in mental disorders (Raybould *et al.*, 1994; Wettstein *et al.*, 1994). The demonstrated presence of CCK_B receptors on enteric neurons indicates that, for a more complete understanding of CCK function in the gastrointestinal tract, research is needed that targets the role of the neural CCK_B receptor subtypes (cf. Enck *et al.*, 1994).

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Chapter 4

CCK_A and CCK_B receptors mediate dissimilar effects of CCK on guinea-pig myenteric neurons

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CCK_A and CCK_B receptors mediate dissimilar effects of CCK on guinea-pig myenteric neurons

Irma W. M. Schutte, Louis M. A. Akkermans, and Alfons B. A. Kroese

Abstract

Intracellular electrophysiological recording was used to study the effects of CCK-8 and its non-sulfated analogue CCK-8NS on 103 myenteric AH neurons of the guinea-pig distal ileum. CCK-8 and CCK-8NS (1-1000 nM) both had an excitatory effect on 48% and an inhibitory effect on 11% of the neurons. The excitatory action was accompanied by a more than 50% reduction (500 nM CCK) of the amplitude and duration of the postspike after-hyperpolarization and a change in membrane resistance. At low concentrations CCK induced a decrease in resistance (to 80% of control; EC_{50} 7 nM; CCK-8NS) and at higher concentrations an increase (to 120% of control; EC_{50} 123 nM; CCK-8NS). The decrease in resistance could be antagonized by the CCK_B receptor antagonist L-365,260 (250 nM), whereas the increase in resistance was antagonized by the CCK_A receptor antagonist L-364,718 (250 nM). The inhibitory action of CCK was accompanied by an enhancement of the after-hyperpolarization and a decrease in membrane resistance (to 60% of control; IC_{50} 75 nM; CCK-8NS). This inhibition was completely antagonized by L-365,260 (250 nM), but not by L-364,718 (250 nM). We conclude that the excitatory effects of CCK on individual myenteric AH neurons are mediated by CCK_A receptors, evoking an increase in membrane resistance, as well as by CCK_B receptors, evoking a decrease in membrane resistance. Thus, the CCK_A and CCK_B receptor subtypes on myenteric AH neurons do not only have different affinities for CCK, but do also mediate excitatory effects through dissimilar ionic channels.

Introduction

Cholecystokinin (CCK) is a small peptide involved in the regulation of several aspects of gastrointestinal function such as gallbladder contraction, gastric emptying and the motility of small intestine and colon (Walsh, 1994). CCK is located in mucosal

endocrine cells in the duodenum and released in response to a meal (Buffa *et al.*, 1976). Further, within the enteric nervous system, CCK released from enteric neurons also functions as a neurotransmitter (Schutte *et al.*, 1997*a*). The predominant and biologically most active form of CCK is the octapeptide CCK-8 (Rehfeld, 1978; Walsh, 1994).

Two different types of CCK receptors, CCK_A and CCK_B, have been described in both the central and peripheral nervous system (Dourish and Hill, 1987; Wank, 1995). These receptor subtypes can be distinguished by their different binding properties of CCK analogues (reviewed by Crawley and Corwin, 1994) and by using selective antagonists, such as L-364,718 (CCK_A; Chang and Lotti, 1986) and L-365,260 (CCK_B; Lotti and Chang, 1989).

CCK is reported to act both directly (Botella *et al.*, 1992) and by neural intermediation on ileal motility (Vizi *et al.*, 1973; Hutchinson and Dockray, 1981). There is indirect evidence that the neurally mediated CCK contraction of smooth muscle cells is mediated by CCK_A as well as CCK_B receptor subtypes. Activation of neural CCK_B receptors in muscle strips produces a transient fast contraction through release of acetylcholine, whereas CCK acting at CCK_A receptors induces an additional slow sustained contraction through the release of Substance P (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992). It is not known if the difference between the CCK-induced contractions is caused by a dissimilarity between the actions mediated by both receptor subtypes, or between the neurons involved.

The main action of CCK on myenteric neurons in the guinea pig ileum is excitatory with a depolarization leading to an increase in action potential activity (Nemeth *et al.*, 1985). We have recently shown that in myenteric S neurons, which are presumably motor- or inter- neurons (Bornstein *et al.*, 1991), both the CCK_A and the CCK_B receptor subtypes are involved in this excitatory response (Schutte *et al.*, 1997*b*). Further, the results of this study suggested that the neural responses mediated by both receptor subtypes differ with respect to their duration. For populations of neurons in the CNS, differences between the effects of CCK mediated by both receptor subtypes have been reported (Dun *et al.*, 1991; Branchereau *et al.*, 1992; Boden and Woodruff, 1994). This does, however, not unequivocally demonstrate a difference between the effects of CCK mediated by both receptors at the single cell level, because these differences may result from dissimilarities in receptor-effector coupling mechanisms between neural populations.

In the present study, the effects of CCK-8 on myenteric AH neurons, which are primary sensory neurons (Kunze *et al.*, 1995) were investigated. The CCK_B

receptor agonist CCK-8NS and the CCK antagonists L-364,718 and L-365,260 were used to characterize the neuronal receptor subtypes. We report here that in individual AH neurons possessing both receptor subtypes, CCK induces two different membrane resistance changes, each of which is mediated by a particular CCK receptor subtype.

Material and Methods

Preparation

Guinea-pigs (200 - 300 g) were stunned by a blow to the head, decapitated and exsanguinated. All procedures were approved by the Wageningen University Committee for Experiments on Animals and were in accordance with the Dutch Law on experimental animals (1987). The methods of dissection were the same as described in a previous paper (Schutte *et al.*, 1997*b*). A conventional myenteric plexus/longitudinal muscle preparation was pinned to Sylgard resin at the glass bottom of a 1.5 ml recording chamber (diameter 21 mm). The chamber was perfused at a rate of 3 ml/min with Krebs buffer, which had the following composition (mM): NaCl 120; KCl 6.0; $MgCl_2$ 1.2; NaH_2PO_4 1.4; $NaHCO_3$ 14.4; $CaCl_2$ 2.5; glucose 12.7; gassed with 95% O_2 - 5% CO_2 and maintained at 36 °C and pH 7.4. Nifedipine (1-2 μM) was added to the perfusion fluid to prevent mechanical activity of the muscle layer.

Electrophysiological recording

The tissue chamber was placed on the fixed stage of a Zeiss UEM microscope equipped with a Hoffman Modulation Contrast system. Individual cells in a ganglion were visualized by a 30x objective (total magnification x600) with a working distance of 10 mm. The ganglion from which recordings were made was immobilized using L-shaped stainless steel wires pressing on the preparation. Intracellular recordings were made using glass electrodes made of borosilicate glass (World Precision Instruments Inc.; 1B100f) on a Brown-Flaming micropipette puller (Sutter Instruments Co.;P-87). The electrodes were bent about 90 degrees at about 6 mm from their tips, filled with 3 M KCl and had a resistance of 60-80 MΩ. Under visual control selected cells were penetrated in the direction perpendicular to the surface of the preparation, by applying an oscillating voltage to the electrode tip.

Potentials were recorded with an electrometer (World Precision Instruments Inc.; Intra 767), by which rectangular current pulses were also injected. Voltage and

current signals were displayed on an oscilloscope. After amplification and low pass filtering (2.6 kHz) each signal was digitized using a 1401plus CED interface (Cambridge Electronic Design Ltd.) at a sampling rate of 5.6 kHz and displayed and stored on a PC.

Drugs and their administration

CCK-8 and CCK-8NS were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of CCK-8 and CCK-8NS were prepared in concentrations of 50 μM in Krebs solution containing bovine serum albumin, aprotonin and bacitracin (each at 0.1%) and stored at -20 °C. The selective CCK_A antagonist L-364,718 and CCK_B antagonist L-365,260, were kindly provided by Dr. V. Lotti (Merck, Sharp & Dohme, Rahway, NJ). L-364,718 and L-365,260 were dissolved in DMSO (99%) and frozen at -20^0 C. Solutions at the desired concentrations were prepared just before application to the superfusion by thawing fresh aliquots in Krebs solution. In the experiments, DMSO never exceeded 0.1% in the perfusion medium. Control experiments showed that 0.1% DMSO did not affect the electrical properties of the neurons.

CCK-8 and CCK-8NS were applied by micro-ejection from fine-tipped pipettes (tip diameter 10 μm) with nitrogen pulses of controlled pressure and duration, or by addition to the superfusion solution in known concentrations. Application by micro-ejection was at intervals exceeding 2 min. CCK-8 and CCK-8NS (1 nM - 1000 nM) were applied in the bath for 1-5 min. Between bath applications, the tissue was kept in drugs-free solution for at least 15 minutes to ensure reproducibility.

In a series of separate experiments, concentration-response curves for the effect of CCK-8NS on membrane resistance were made by adding increasing concentrations of CCK-8NS to the tissue chamber at intervals of 3 min. (see Fig. 3). The membrane resistance was measured during the last 30 s of each 3 min period, when a plateau was achieved in the cell response. Subsequently, concentration-response curves of CCK-8NS were measured in the presence of a CCK receptor antagonist, starting 30 min after the antagonist application.

Classification of cells

Excitable cells were classified electrophysiologically "on line" by using a criterion of which we have recently shown that this distinguishes S and AH neurons unequivocally (Schutte *et al.*, 1995). Cells in which the action potential evoked by current injection showed a pronounced inflexion (shoulder) on the falling phase, were

classified as AH neurons, the others as S neurons (Bornstein *et al.*, 1994; Schutte *et al.*, 1995). The classification was confirmed by "off line" scrutinizing of the action potential shape in the digitized datafiles.

Data analysis

The data were analyzed "off line" using standard CED SPIKE2 software and computer programs written in the CED SCRIPT language. The membrane resistance of the cells was measured from the voltage response to hyperpolarizing current pulses (20 ms; 4/s) through the cell membrane, by averaging 40 responses. All values are given as means ± standard error of the mean (S.E.M.).

Results

Results were obtained from 103 AH neurons in preparations from 90 guinea-pigs. The neurons had a membrane potential of -64 ± 0.9 mV and membrane resistance of 93 ± 6.3 MΩ. The impalements were maintained for 30 min to three hours. Application of CCK (CCK-8 or CCK-8NS) had an excitatory effect on 50 of 103 (48%) AH neurons and an inhibitory effect on 11 of 103 (11%) neurons. No differences were found in electrical properties between neurons that showed a response upon CCK administration and those that did not. The effects of CCK applied by micro-ejection (50 μM in pipette; 10-60 ms ejection) on the excitability of the neurons reached a maximum within 10 s and than gradually diminished, due to the decreasing CCK concentration in the bath. CCK application in the superfusion (1 nM - 1000 nM; 1-5 min) evoked longlasting changes in excitability, which were completely reversible. The response persisted as long as CCK was in the bath, and no signs of desensitation were observed. The excitatory and inhibitory effects of CCK persisted in the presence of TTX (10 nM; n=7), indicating a direct action on the neurons (c.f. Schutte *et al.*, 1997*b*).

Excitatory effects of CCK

Effects on after-hyperpolarization

The excitatory effect of both forms of CCK (CCK-8, n=42; CCK-8NS, n=15) was mainly apparent on the, for AH neurons characteristic, longlasting (> 4 s) postspike after-hyperpolarization. Application of CCK by micro-ejection caused a reversible shortening of the duration of the after-hyperpolarization (Fig. 1*A*),

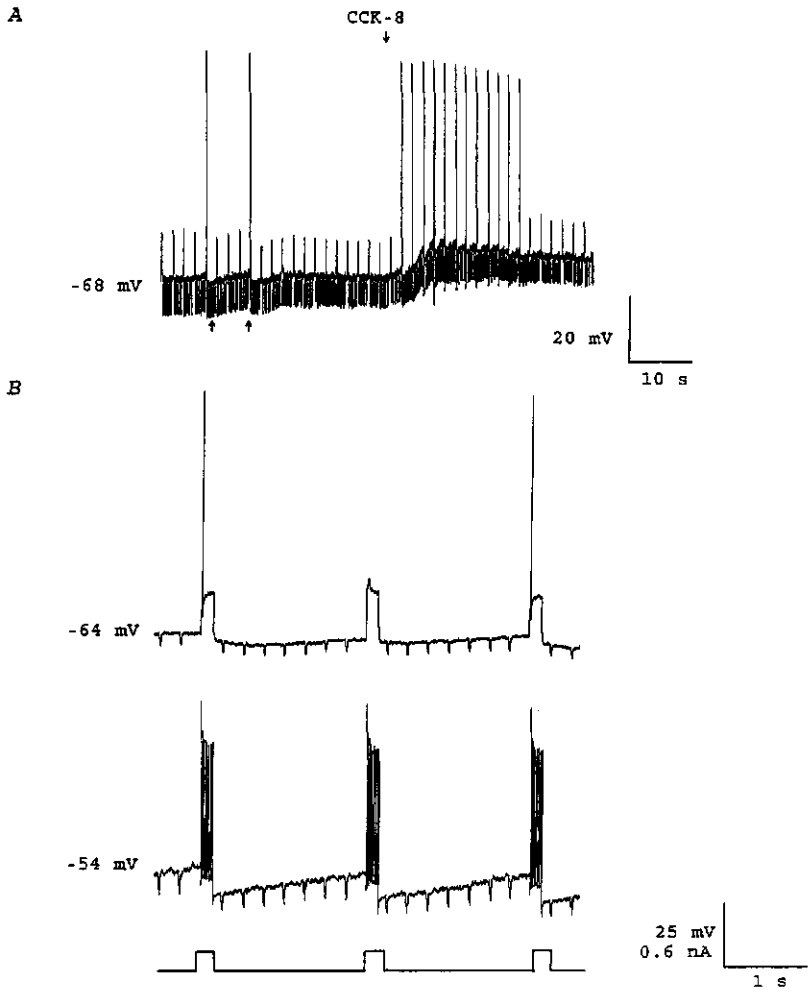

Figure 1. Excitatory action of CCK-8 on myenteric AH neurons. **A:** Intracellular depolarizing current pulses (upwards deflexions; 0.5 Hz) evoked an action potential followed by a prolonged hyperpolarization (indicated by arrows) associated with decreased input resistance (downwards deflexions) and diminished excitability. After micro-ejection of CCK-8 (50 μM; 50 ms), the neuron depolarized, the hyperpolarization was shortened and the excitability was augmented. Membrane potential is indicated (- 68 mV). **B: Top trace**, under control conditions injection of a depolarizing current pulse (see bottom trace; 150 ms; 0.5 Hz; 0.2 nA) induces a single action potential followed by an after-hyperpolarization. **Middle trace**, after application of CCK-8 (50 μM; 50 ms), the duration of the after-hyperpolarization was reduced and multiple spikes were evoked during each depolarizing current pulse. Membrane potential is indicated. **Bottom trace**, depolarizing current pulses.

accompanied by a higher frequency of action potential discharge upon depolarizing current pulses (Fig. 1*B*). In some neurons, CCK-8 induced a small depolarization (4.0 ± 6.5 mV; *n*=25), but in other neurons no change in membrane potential was detected (*n*=13) or even a small hyperpolarization (4.1 ± 1.2 mV; *n*=4). When the cells were held at resting membrane potential by current clamp, CCK-8 (100 nM) reduced the amplitude of the after-hyperpolarization to 88 ± 3% and the total duration to 40 ± 5% of their respective control values (*n*=4). A higher concentration of CCK-8 (500 nM), reduced the amplitude to 48 ± 2% and the duration to 33 ± 7% of their respective control values (*n*=4). In contrast to S neurons (Schutte *et al.*, 1997*b*), the increase in excitability was never accompanied by spontaneous action potential discharge or induction of fEPSPs. Application of CCK did not affect the threshold, amplitude or duration of the action potentials.

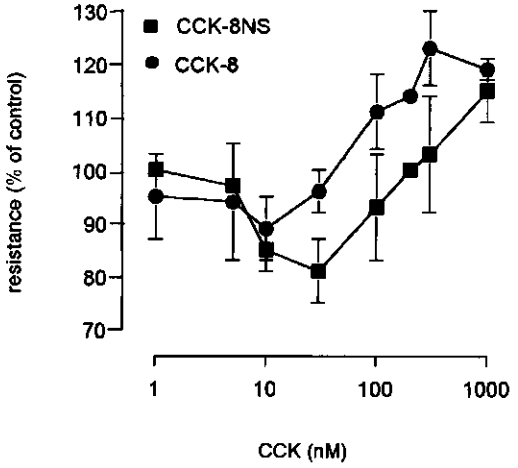

Figure 2. Concentration-response relationship for effect of CCK-8 and CCK-8NS on membrane resistance in myenteric AH neurons. Resistance is expressed as % of control. Number of neurons for each datapoint is 7-8. Vertical bars represent S.E.M..

Effects on membrane resistance

The effects on membrane resistance evoked by CCK-8 application to the superfusion (1 nM - 1000 nM) were investigated in 24 neurons. Application of CCK-8 induced a concentration dependent effect on membrane resistance which was biphasic (Fig. 2). A small decrease in membrane resistance was induced at low concentrations of CCK-8 (10 nM) and an increase at higher (>100 nM)

mainly seen as a reduction of the CCK-8 induced inhibition of the after-hyperpolarization and of the action potential frequency in response to depolarizing constant current pulses. Figure 5 shows an example of a neuron with an excitatory response to CCK-8 and CCK-8NS, and the effects of the antagonists. Application of CCK-8 by micro-ejection to this neuron caused a depolarization associated with an increase in resistance to 106% of control (Fig. 5A), as CCK-8NS evoked a decrease in resistance to 89% of control (Fig. 5B). In the presence of L-365,260, CCK-8 evoked a small depolarization (3 mV) accompanied by an increase in resistance (to 108% of control; Fig. 5C), whereas in the presence of L-364,718 a decrease in resistance (to 92% of control; Fig. 5D) was induced. Thus, application of the antagonists affected the CCK-8-induced resistance changes in a way comparable to that observed with CCK-8NS.

The duration of the excitatory effects of CCK on the AH neurons was not different for the two receptor subtypes, contrary to that observed in S neurons (Schutte *et al.*, 1997*b*).

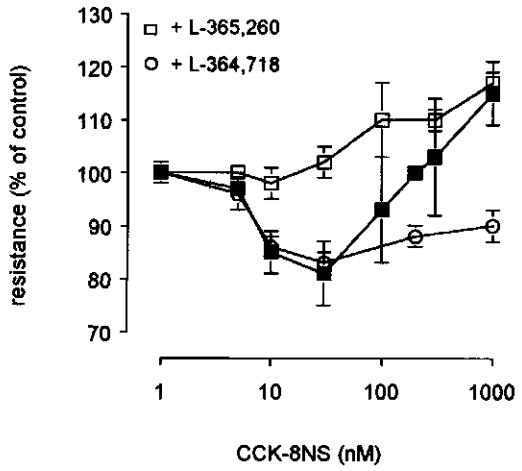

Figure 4. Effects of CCK receptor antagonists on the concentration-response curve of CCK-8NS of myenteric AH neurons. The concentration-response curve of the effect of CCK-8NS on membrane resistance is biphasic. L-365,260 (250 nM) antagonized the decrease in resistance, whereas L-364,718 (250 nM) antagonized the increase. Membrane resistance is depicted in % of control. Vertical bars represent S.E.M.. Number of neurons for each data point is 7-8.

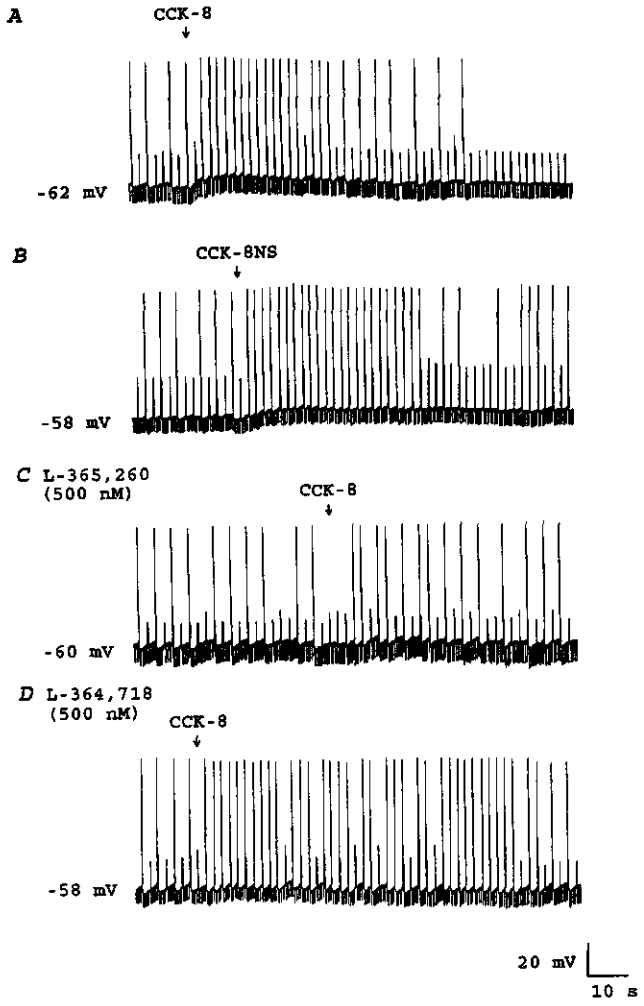

Figure 5. Effects of CCK-receptor antagonists on the response to CCK-8 of an AH neuron. **A:** Application of CCK-8 by micro-ejection (50 μM; 50 ms) suppressed the after-hyperpolarization leading to an enhanced excitability accompanied by a depolarization (5 mV) and an increase in membrane resistance to 106% of control. **B:** Application of CCK-8NS by micro-ejection (50 μM; 50 ms) evoked an excitatory response comparable to that evoked by CCK-8 application, accompanied by a depolarization (4 mV). The membrane resistance, however, decreased to 89% of control. **C:** In the presence of L-365,260 (500 nM, micro-ejection of CCK-8 evoked a small depolarization (3 mV) accompanied by an increase in resistance to 108% of control. **D:** During application of L-364,718 (500 nM), micro-ejection of CCK-8 evoked a depolarization of 4 mV accompanied by a decrease in membrane resistance to 90% of control. On the left the membrane potential is indicated.

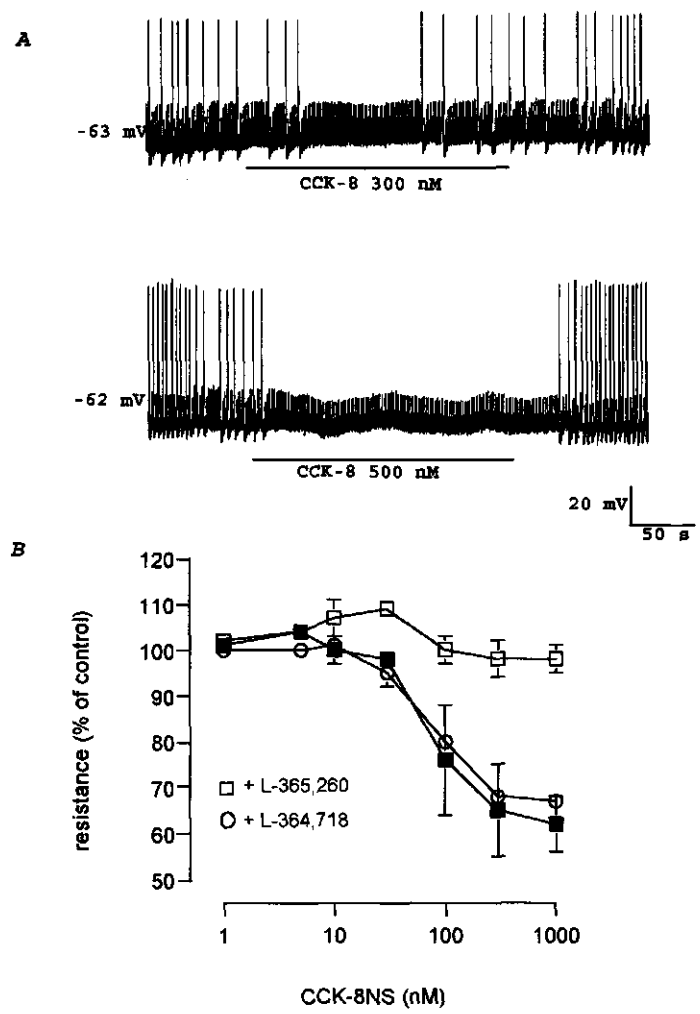

Figure 6. Inhibitory effect of CCK-8 on a myenteric AH neuron. **A: Top trace**, Application of CCK-8 (300 nM) evoked an inhibition of the action potential response to depolarizing current pulses, accompanied by a decrease in membrane resistance to 83% of control. **Bottom trace**, During superfusion of 500 nM CCK-8 the neuron did not discharge action potentials upon constant depolarizing current pulses (decrease in membrane resistance to 85% of control). Application of CCK-8 is indicated by bar. On the left the membrane potential is indicated. **B:** Concentration-response relations of the inhibitory effect of CCK. CCK-8NS (n=3) evoked a concentration-dependent decrease in membrane resistance. L-365,260 (250 nM) antagonized completely the CCK-8NS induced decrease in membrane resistance, whereas L-364,718 had no effect. Membrane resistance is depicted in % of control. Vertical bars represent S.E.M..

Inhibitory effects of CCK

In 11 of 103 (11%) AH neurons, application of CCK-8 (n=8) or CCK-8NS (n=3), without altering the resting membrane potential, reduced the frequency of action potential discharge on depolarizing current pulses (Fig. 6*A*), and enhanced the duration of the after-hyperpolarization. This inhibitory action of CCK-8 was concentration dependent and reversible (Fig. 6*A*). The duration and amplitude of the action potentials were not changed, but the threshold for action potential discharge changed from -48 ± 2 mV to -40 ± 3 mV (CCK-8 500 nM).

Associated with the inhibitory response to CCK-8 was a decrease in membrane resistance (micro-ejection; to 91 ± 7% of control). The resistance decreased with increasing concentrations of CCK-8 in the bath until 83% of control at 300 nM CCK-8 (IC_{50} 20 nM). Similarly, in the three neurons to which CCK-8NS was applied, a concentration dependent decrease in membrane resistance to 62% of control (1000 nM) was observed. Data pooled from these neurons are depicted in figure 6*B* (IC_{50} 75 nM; CCK-8NS). The response was completely antagonized by L-365,260 (250 nM) as L-364,718 (250 nM) had no effect, showing that the decrease in membrane resistance involved in the inhibitory effects of CCK is mediated by CCK_B receptors.

Discussion

The predominant response of the myenteric AH neurons to CCK was a slow excitation mediated in each neuron by postsynaptic CCK_A and CCK_B receptor subtypes. In a small portion of the neurons an inhibitory response to CCK was encountered, which was mediated through the CCK_B receptor subtype.

Excitatory response to CCK

Several findings in this study demonstrate that in all CCK responsive myenteric AH neurons, different resistance changes are mediated by CCK interacting with the two receptor subtypes.

First, the concentration-response curves for the effects of CCK-8 and CCK-8NS on the resistance show that the direction of the resistance change is concentration dependent. This concentration-dependency relates to the about 10,000 times higher affinity of CCK-8NS for the CCK_B than for the CCK_A receptor (Lucaites *et al.*, 1991). So, CCK-8NS in low concentrations most likely activates CCK_B receptors, inducing a decrease in resistance, as CCK-8NS in high concentrations also

Evidence for a role of cholecystokinin as neurotransmitter in the guinea-pig enteric nervous system

Irma W. M. Schutte, Louis M. A. Akkermans, and Alfons B. A. Kroese

Abstract

Intracellular recordings were made of neurons in the myenteric plexus of the guinea-pig distal ileum. Slow excitatory postsynaptic potentials (sEPSPs) were evoked by electrical stimulation of an interganglionic fibre tract. The effect of cholecystokinin (CCK) receptor antagonists on the sEPSPs was investigated in 11 neurons. Application of the CCK receptor antagonists L-364,718 and L-365,260 (each 250 nM) markedly attenuated the sEPSPs in fiveof 11 neurons. The amplitude of the sEPSP reduced from 15 ± 3 mV to 7 ± 2 mV and the change in membrane resistance during the sEPSP was reduced from 28 ± 9 MΩ to 11 ± 8 MΩ. In six of 11 neurons the CCK antagonists had no effect on the sEPSPs. The results provide evidence that neurally released CCK is involved in the mediation of sEPSPs in some enteric neurons.

Introduction

Cholecystokinin (CCK) is a neuropeptide with a widespread distribution throughout the central nervous system (Crawley and Corwin, 1994) and the gastrointestinal tract (Walsh, 1994). The gut hormone CCK is released after a meal from endocrine cells in the duodenum (Buffa *et al.*, 1976). The release of CCK induces a variety of effects on gastrointestinal motility and secretion, which are partly nerve mediated (Walsh, 1994). Application of CCK evokes slow, mainly excitatory, responses in myenteric neurons of the guinea pig ileum (Nemeth *et al.*, 1985; Schutte *et al.*, 1996, 1997), which are mediated by both CCK_A and CCK_B receptor subtypes (Schutte *et al.*, 1996, 1997)

In the central nervous system, CCK functions as a neurotransmitter or neuromodulator (Crawley and Corwin, 1994; Wettstein *et al.*, 1994). CCK is considered a putative neurotransmitter in autonomic ganglia (Schumann and Kreulen, 1986) and in the enteric nervous system of the gut (Crawley and Corwin, 1994;

Dockray, 1994; McConalogue and Furness, 1994; Walsh, 1994; Wood, 1994). This is based on the immunohistochemical evidence for the localization of CCK in enteric neurons and nerve fibers (Furness *et al.*, 1984, 1995; Larsson and Rehfeld, 1979; Schultzberg *et al.*, 1980). CCK is thought to be involved as a neurotransmitter in peristalsis (Walsh, 1994) and recently a role of CCK as a neurotransmitter in the regulation of canine colonic motility has been suggested (Karaus and Niederau, 1995). A neurotransmitter function of CCK would be in accordance with the fact that application of CCK to enteric neurons causes responses which mimic the slow excitatory postsynaptic potentials (sEPSPs) evoked by presynaptic electrical stimulation (Wood, 1994).

The sEPSP consists of a longlasting membrane depolarization which is associated with an increase or decrease in membrane resistance, to which several types of ionic channels may contribute (Schutte *et al.*, 1996; Surprenant, 1994; Wood, 1994) and a discharge of action potentials. Many different neurotransmitters are involved in the generation of sEPSPs (McConalogue and Furness, 1994; Surprenant, 1994; Wood, 1994). For acetylcholine, 5-HT and substance P an excitatory neurotransmitter function has been demonstrated unequivocally (Wood, 1994). The evidence for a role of CCK as neurotransmitter in the enteric nervous system is, however, not very strong and incomplete (Dockray, 1994; McConalogue and Furness, 1994; Walsh, 1994). The aim of the present study is to determine if neuronally released CCK is involved in the electrically evoked sEPSP, by investigating the effects of CCK receptor antagonists on the sEPSPs.

Material and Methods

Intracellular recordings were made from neurons in isolated preparations of the myenteric plexus of the guinea-pig distal ileum (200-300 g). Standard techniques, previously described in detail (Schutte *et al.*, 1995, 1997), were used for dissection of the tissue and recording. All procedures were approved by the Wageningen University Committee for Experiments on Animals. The preparations were maintained in a low volume chamber that was perfused at a rate of 3 ml/min with Krebs solution at 37° C and gassed with 95% O_2-5% CO_2. Nifedipine (1-2 μM) was added to the perfusion fluid to prevent mechanical activity of the muscle layer. Intracellular recordings were made using glass electrodes (3 M KCl; 60-80 MΩ). A stainless steel electrode (tip diameter 10 μm) was used to stimulate an interganglionic fibre tract

connected to the ganglion. The distance between the electrode and the ganglion was at least 200 μm. Slow EPSPs were evoked by extracellular current pulses of 0.15 ms duration (20 Hz, 1 s) produced by a stimulus isolation unit. Stimuli were applied only when the membrane potential had returned to the original membrane potential after a preceding stimulation. Neurons in which the action potential evoked by current injection showed a pronounced inflexion (shoulder) on the falling phase, were classified as AH neurons, the others as S neurons (Bornstein *et al.*, 1994; Schutte *et al.*, 1995).

All recorded signals were digitized and were analysed using standard CED (Cambridge Electronic Design Ltd) software and computer programs written in the CED SCRIPT language. The membrane resistance of the neurons was measured from the voltage response to hyperpolarizing current pulses through the cell membrane, by averaging at least 10 responses. The time course and maximum of changes in membrane potential and membrane resistance during the sEPSP were determined by measuring these parameters every few (1-10) seconds. For each neuron, the mean of the maximum values of these parameters was obtained from -on average- three sEPSPs per experimental condition. The selective CCK_A antagonist L-364,718 (Chang and Lotti, 1986) and CCK_B antagonist L-365,260 (Lotti and Chang, 1989) were dissolved in DMSO (99%) and frozen at -20° C. Solutions of the antagonists were prepared at a concentration of 250 nM just before application to the superfusion by thawing fresh aliquots in Krebs solution. DMSO (bath concentration always < 0.1%) had no effect on the electrical properties and excitability of the neurons. The antagonists were used simultaneously, because CCK-responsive myenteric neurons do possess either the CCK_A or the CCK_B, or both receptor subtypes (Schutte *et al.*, 1996; 1997).

Results

The results are based on intracellular recordings from 11 neurons (seven AH and four S) in 10 preparations. The neurons had a membrane potential of 60 ± 2 mV (mean ± S.E.M.) and a membrane resistance of 72 ± 14 MΩ. All neurons showed a constant membrane potential and resistance throughout the experiment and showed reproducible sEPSPs. The amplitude of the evoked sEPSPs varied considerably among individual neurons; ranging from 8 to 25 mV, with a mean of 14 ± 2 mV. The time course of the depolarization was longlasting, ranging from 20 s to 5 min, with a

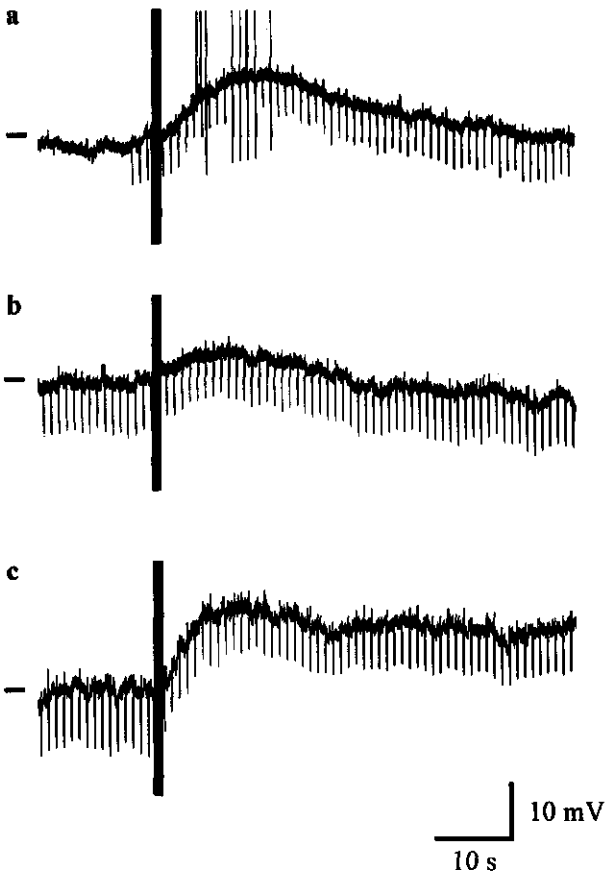

Figure 1. Antagonism of the sEPSP by CCK receptor antagonists. **A:** Under control conditions electrical stimulation (bar; 20 Hz, 1 s) induces a sEPSP with a few action potentials (truncated). The amplitude of the sEPSP is 11 mV (rmp -54 mV; indicated) and the membrane resistance (vertical deflections; see text) decreases from 63 MΩ to 45 MΩ. **B:** In presence of the CCK receptor antagonists (L-364,718 and L-365,260; 250 nM each) the amplitude of the sEPSP is reduced to 5 mV without any change in membrane resistance (66 MΩ). **C:** Recovery of the sEPSP after washout of the antagonists. The amplitude is 13 mV and the membrane resistance decreases from 69 MΩ to 49 MΩ.

mean of 157 ± 40 s. The maximal depolarization was found at 24 ± 7 s after stimulation. In nine (five AH, four S) neurons, the depolarization was accompanied by an increase in membrane resistance, due to closure of potassium channels (Surprenant, 1994). In two AH neurons a decrease in resistance occurred, presumably due to opening of nonselective cation channels (cf. Surprenant, 1994;

Schutte *et al.*, 1996).The average change in membrane resistance, for the increases and decreases together, was 31 ± 6 MΩ, which is about 43 % of the resting membrane resistance of the neurons. Dose-response studies have shown that the antagonists at 250 nM inhibit the action of CCK on myenteric neurons (Schutte *et al.*, 1996, 1997). Superfusion of the antagonists induced no changes in the resting membrane potential (61 ± 2 mV re control 60 ± 2 mV) or the membrane resistance (67 ± 10 MΩ re control 72 ± 14 MΩ) of the neurons.

Table 1. Effect of CCK antagonists on sEPSP properties of 11 neurons.

	control	CCK antagonists
attenuation of sEPSP (n=5):		
depolarization (mV):	15 ± 3	7 ± 2 *
resistance change (MΩ):	28 ± 9	11 ± 8 *
no effect on sEPSP (n=6):		
depolarization (mV):	13 ± 4	14 ± 4
resistance change (MΩ):	34 ± 8	35 ± 6

Within a row, * indicates values (mean ± S.E.M) that are significant different from control (Wilcoxon; $P < 0.05$). The control values for depolarization and resistance change of the two groups were not significantly different (Mann-Whitney; $P > 0.05$).

Superfusion of the preparations with the antagonists was found to inhibit markedly the sEPSPs in five (four AH, one S) of 11 neurons. In these neurons, the antagonists significantly reduced (Table 1) the amplitude of the sEPSP as well as the change in membrane resistance. An example is given in Figure 1. In the five neurons, the amplitude of the sEPSP was reduced from 15 ± 3 mV to 7 ± 2 mV and the change in resistance from 28 ± 9 MΩ to 11 ± 8 MΩ (Table 1). The sEPSPs recovered after washout of the antagonists (Fig. 1).

In six (three AH, three S) of 11 neurons, application of the antagonists had no effect on the sEPSPs (see Table 1). For the 11 individual neurons, the effects of the

antagonists on the amplitude and resistance change of the sEPSP are shown in Figure 2, expressed as percentage of control values. Statistical analysis confirmed the presence of two populations of neurons, in one of which the sEPSP was inhibited by the antagonists (see legend Fig. 2).

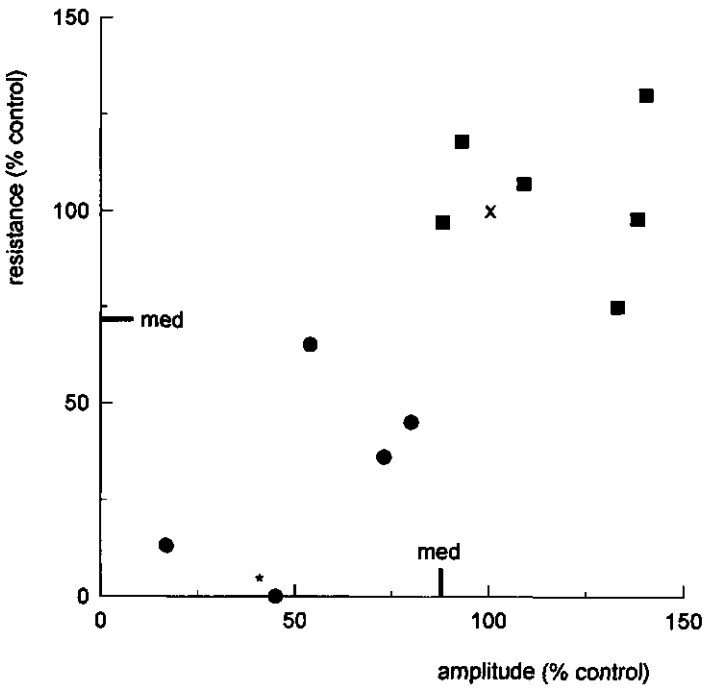

Figure 2. Effect of CCK receptor antagonists on sEPSP amplitude and resistance change of 11 neurons, expressed as percentage of control (X). The median values for amplitude and resistance of the 11 neurons are indicated along the axis. The symbols are unequally distributed over the 4 quadrants determined by the 2 medians (*Chi*-square test; Fisher two-tailed P = 0.002). This leads to the conclusion that there are 2 populations of neurons, which responded differently to the antagonists. In 5 neurons (●) the antagonists inhibited the sEPSP and in 6 neurons (■) the sEPSP was not affected. This is in accordance with the results of the analyses based on the mean values (Table 1). Of the 2 AH neurons in which the sEPSP was accompanied by an decrease in resistance, one was affected by the antagonists. * labels the neuron shown in Fig.1. The values for amplitude and resistance showed a high correlation (coefficient 0.79 with two-tailed $P < 0.05$).

Discussion

The antagonists L-364,718 and L-365,260 are highly selective for respectively the CCK_A and CCK_B receptor subtype and there is evidence that these antagonists do not interfere with other receptors (Chang and Lotti, 1986; Lotti and Chang, 1989). Thus, the observed suppression of the sEPSP can be considered to result from a blockade of CCK receptors in the pathway between electrical stimulus and recorded sEPSP. Even if not only mono-synaptic but also multi-synaptic pathways are involved in the induction of the sEPSP, the results do indicate that one of the released neurotransmitters in the pathway is CCK. Paracrine release of CCK by the stimulation seems excluded, because the mucosa in which the endocrine CCK cells are located (Buffa *et al.*, 1976) is not part of the isolated preparation. This suggests that the presynaptic electrical stimulation of the nerve fibers causes neural release of CCK, which mediates the sEPSP.

Most likely, each sEPSP results from the simultaneous release of many neurotransmitters, because the electrical stimulation of the interganglionic fibre tract will evoke action potential activity in the hundreds of nerve fibers it contains. Further, plurichemical transmission is quite common in the enteric nervous system and two or more transmitter substances are usually colocalized within single neurons (Dockray, 1994; Furness *et al.*, 1995; McConalogue and Furness, 1994). Such a simultaneous release of many neurotransmitters in response to the electrical stimulus might explain the finding that the sEPSPs were only partly reduced by the CCK antagonists.

The lack of effect of the antagonists on sEPSPs of particular neurons could result from such a massive release of other neurotransmitters upon stimulation or it may be that not all neurons receive innervation from CCK neurons. This would be in accordance with the immunohistochemical data, which indicate that CCK is localized in a relatively small population (about 5%) of myenteric neurons (Furness *et al.*, 1984; Schultzberg *et al.*, 1980). Moreover, receptors for CCK seem to be localized in a subpopulation of neurons, since application of CCK to myenteric neurons evokes excitatory responses in 85% of S neurons and 50% of AH neurons (Nemeth *et al.*, 1986; Schutte *et al.*, 1997).

The conclusion that CCK can be released from myenteric neurons suggests a neurotransmitter function for CCK. For CCK in the enteric nervous system, the main criteria for a function of a peptide as a neurotransmitter (Dockray, 1994) are now

fulfilled: (1) there is evidence for presence of CCK in enteric neuronal cell bodies (Furness *et al.*, 1984, 1995; Larsson and Rehfeld, 1979; Schultzberg *et al.*, 1980); (2) CCK application evokes sEPSP responses in the neurons (Nemeth *et al.*, 1986; Schutte *et al.*, 1996, 1997), which indicates the presence of functional receptors; (3) CCK appears to be released from neurons upon nerve stimulation (present experiments). More detailed studies are necessary to determine the function of the neurotransmitter CCK in the myenteric plexus and the functions of the neurons involved.

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It was concluded that the shoulder of the action potential can be used as a single criterion to distinguish "on line" S and AH neurons unequivocally. The advantages of this classification above other existing classification schemes are that the excitable cells can be distinguished "on line", using only one criterion and that it is highly selective and consistent.

In **Chapter 3**, the actions of CCK-8 on S neurons were determined. S neurons are thought to function as inter- or motor-neurons (Bornstein, 1994). Application of CCK-8 caused longlasting increases in excitability of almost all S neurons, apparent as spontaneous action potential discharge or an increase in action potential discharge during depolarizing current pulses. The increase in excitation was accompanied by a depolarization and an increase in membrane resistance, which were concentration-dependent (1-1000 nM CCK-8). CCK-8 was found to have a direct action on the recorded neurons and no presynaptic effects were encountered.

The CCK receptor subtypes involved in the excitatory action of CCK were characterized using selective receptor antagonists. Application of the selective CCK_A antagonist L-364,718 (100-500 nM), antagonized the CCK-8 induced response in part of the neurons, as the CCK_B antagonist L-365,260 (500 nM) had no effect, indicating that these neurons possessed exclusively the CCK_A receptor. In other neurons, the CCK-8 induced responses were antagonized by L-365,260 (100-500 nM), as L-364,718 (500 nM) had no effect on these neurons, indicating that these neurons possessed exclusively the CCK_B receptor. In a third group of neurons, each antagonist partly inhibited the CCK-8 evoked application, and application of both antagonists (500 nM) caused a complete blockade of the response, indicating that these neurons possessed both receptor subtypes. The presence of CCK_B receptors in the enteral nervous system had been suggested based on in vitro contraction studies (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992) using longitudinal muscle strips, but this is the first evidence that some enteric neurons actually possess the CCK_B receptor subtype. The occurrence of both CCK-8 receptor subtypes on individual neurons had till now only been reported for several brain regions (Boden and Woodruff, 1994).

Activation of these different receptor subtypes was always associated with an increase in membrane resistance, indicating that the underlying ionic mechanism was similar for both receptor subtypes. The excitatory responses to CCK-8 mediated by the two receptor subtypes were however different. Only in neurons in which CCK-8 acted through the CCK_A receptor subtype, abundant spontaneous action potential

activity was observed upon application of CCK-8. Also the duration of the response mediated by the CCK_A receptor was longer than that mediated by the CCK_B receptor. This might explain the observations from in vitro contraction studies that CCK acting on CCK_B receptors evoked a short contraction as contractions mediated by CCK_A receptors were sustained (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992).

It was concluded that the excitatory action of CCK-8 on myenteric S neurons is mediated not only by CCK_A, but also by CCK_B receptor subtypes, and was different for both receptor subtypes with respect to action in time. Some neurons possessed exclusively the CCK_A or the CCK_B receptor subtype, but others possessed both subtypes.

In **Chapter 4**, the actions of CCK-8 and its non-sulfated analogue CCK-8NS (a CCK_B agonist) on myenteric AH neurons were investigated. Only part of the AH neurons showed an effect upon CCK-8 or CCK-8NS application, indicating that only a subpopulation of these neurons possessed CCK receptors. About half of the AH neurons showed a slow excitatory action upon CCK application, as 11% showed a longlasting inhibitory response.

The excitatory response was seen as a suppression of the post-spike after-hyperpolarization, a small depolarization and a change in membrane resistance. No spontaneous action potential discharge was observed after CCK application in AH neurons in contrary to the excitatory response observed in some S neurons.

All AH neurons that responded upon CCK application with an excitatory action were endowed with both CCK_A and CCK_B receptor subtypes. Excitatory responses were associated with either decreases or increases in membrane resistance, which were concentration-dependent and linked to the receptor subtypes. Low concentrations of CCK evoked a decrease in membrane resistance (to 80% of control; EC_{50} 7nM; CCK-8NS), as higher concentrations evoked an increase in resistance (to 120% of control; EC_{50} 123 nM; CCK-8NS). The decrease in resistance could be antagonized by L-365,260 (250 nM), but not by L-364,718 (250 nM), indicating that the decrease was caused by CCK acting at CCK_B receptors. Using the antagonists, the increase was shown to be mediated by CCK acting at CCK_A receptors. These differences in resistance changes linked to the receptor subtypes are probably caused by different underlying ionic mechanisms.

The inhibitory response to CCK was seen as a prolongation of the after-hyperpolarization accompanied by a decrease in resistance to 60% of control (IC_{50} 75 nM; CCK-8NS). This response was shown to be mediated by the CCK_B antagonist.

So, CCK acting at CCK_B receptors evoked both inhibitory and excitatory responses, in both of which the underlying mechanism was a decrease in membrane resistance.

It was concluded that the predominant effect of CCK on myenteric AH neurons was a slow excitation mediated by both CCK_A and CCK_B receptor subtypes. These AH neurons were all endowed with both CCK receptor subtypes, showing a different affinity for CCK and mediating the excitatory effects of CCK in a dissimilar way. In low concentrations, excitatory effects of CCK were mediated by the CCK_B receptor resulting in a decrease in membrane resistance. Higher concentrations showed an additional response of CCK via the CCK_A receptor, resulting in an increase in resistance. In a small population of neurons, CCK acting through the CCK_B receptor evoked an inhibitory response.

In **Chapter 5**, the possible role of CCK as a neurotransmitter involved in the mediation of slow synaptic excitation (sEPSP) was investigated. Therefore the effects of the CCK antagonists on electrically evoked sEPSPs were investigated. Slow EPSPs were evoked by electrical stimulation of an interganglionic fibre tract. The sEPSPs consisted of a longlasting membrane depolarization associated with a change in membrane resistance. Application of L-364,718 and L-365,260 (each 250 nM) markedly attenuated the sEPSPs in part of the tested neurons, indicating that CCK was involved in mediation of these sEPSPs. Both the amplitude and the change in membrane resistance were suppressed.

The finding that the sEPSPs were only partly reduced, and not completely suppressed by the CCK antagonists can be explained by simultaneous release of many neurotransmitters, because electrical stimulation of interganglionic fibre tracts will stimulate many nerve fibers. In the enteral nervous system, two or more transmitters are usually colocalized within one neuron and co-released (McConalogue and Furness, 1994). The lack of effect of the antagonists on part of the sEPSPs can be explained by the fact that CCK is only localized in a small population (5%) of myenteric neurons (Furness *et al.*, 1984), and that receptors for CCK are located in a subpopulation of neurons (see chapters 3 and 4).

It was concluded that neurally released CCK is involved in the mediation of sEPSPs in some enteric neurons. The main criteria for a function of CCK as a neurotransmitter are now fulfilled: (a) CCK is present in neurons (Furness *et al.*, 1984); (b) application of CCK mimics the sEPSP (this thesis, Nemeth *et al.*, 1985); (c) CCK is released from neurons upon nerve stimulation (this thesis).

Conclusions

In summary, the conclusions of the investigations were:

1. The two main types of excitable myenteric neurons, AH and S, could be classified "on line" based on the difference in shape of their action potentials. This classification appeared to be highly selective as well as consistent.

2. The effects of CCK on S and AH neurons were different and can be summarized as follows:

* CCK had only excitatory effects on S neurons, whereas both excitatory and inhibitory effects were encountered in AH neurons.
* Almost all S neurons responded upon CCK application, as only a subpopulation of AH neurons did.
* S neurons possessed either CCK_A, CCK_B or both receptor subtypes, while all AH neurons that responded with an excitatory action upon CCK application were endowed with both CCK receptor subtypes.
* In S neurons, the excitatory response mediated by either CCK receptor subtype was always associated with an increase in resistance, but was different in appearance and action in time. CCK acting at CCK_A receptors evoked a longer lasting response than CCK acting at CCK_B receptors. Thus, the actual response of the S neurons was dependent on which receptor subtype the cell possessed.
* In AH neurons, the excitatory effects mediated by both CCK receptor subtypes were mediated in a dissimilar way. In low concentrations, CCK acting at CCK_B receptors evoked a decrease in membrane resistance, whereas higher concentrations showed an additional response of CCK acting at CCK_A receptors resulting in an increase in resistance. Thus, the actual response of AH neurons was dependent on the local concentration of CCK.

3. Neurally released CCK is involved in the mediation of electrically evoked sEPSPs in some enteric neurons. The main criteria for CCK as a neurotransmitter involved in the mediation of sEPSPs in the enteral nervous system are now fulfilled.

elektrische en synaptische eigenschappen van cellen gemeten worden die informatie geven over het functioneren van de cellen. De effecten van CCK op de S en AH neuronen alsmede de betrokken receptoren werden gekarakteriseerd. Tevens werd een mogelijke rol van CCK als neurotransmitter, betrokken in de generatie van langzame synaptische signalen (sEPSPs), onderzocht.

Overzicht van de studies

Het voornaamste doel van het in **hoofdstuk 2** beschreven onderzoek was om te onderzoeken of het mogelijk is om de AH en S neuronen op grond van één enkel criterium, namelijk het al of niet voorkomen van een schouder op de actiepotentiaal, electrofysiologisch te karakteriseren. Intracellulaire afleidingen van cellen werden gemaakt onder visuele controle. Elektrisch exciteerbare cellen waarvan de actiepotentiaal een schouder vertoonde ten gevolge van calcium instroom (AH cellen) werden onderscheiden van cellen waar dat niet het geval was (S cellen). Een derde klasse werd gevormd door niet-exciteerbare cellen (NS-cellen). De elektrische en synaptische eigenschappen van de verschillende celtypen, zoals membraan potentiaal en membraan weerstand, bleken significant te verschillen.

De naam AH neuron refereert aan de na-hyperpolarisatie (after-hyperpolarization) na een actiepotentiaal. Gedurende de na-hyperpolarisatie zijn de AH neuronen relatief inexciteerbaar. S neuronen vertonen zo'n na-hyperpolarisatie niet en kunnen continue actiepotentialen afgeven. Tijdens de experimenten bleek in een deel van de AH neuronen de na-hyperpolarisatie onderdrukt te zijn, en vertoonde het gedrag van de AH neuronen een grote gelijkenis met dat van de S neuronen. De onderdrukking van de na-hyperpolarisatie was waarschijnlijk het gevolg van afgifte van neurotransmitters die een langzame synaptische response veroorzaken in de cellen. De schouder op de actiepotentiaal was echter altijd zichtbaar en bleek een betrouwbaar criterium om AH van S cellen te onderscheiden.

In dit hoofdstuk werd tevens onderzocht of er een verband bestond tussen electrofysiologisch gedefinieerde celtypen enerzijds en de lokatie binnen het ganglion van de cellen en de grootte van het celsoma anderzijds. Via foto's van de cellen in de ganglia werden van alle electrofysiologisch gekarakteriseerde cellen de grootte van het soma en de lokatie in het ganglion bepaald. De resultaten toonden aan dat de somagrootte van de drie typen cellen significant verschillend was. De AH cellen waren het grootst (1290 μm^2) en hadden een ovaal gevormd celsoma. De S cellen

vertoonden een grote variatie in vorm en waren kleiner (760 μm^2) in vergelijking met de AH cellen. De NS cellen bleken kleine ronde cellen (430 μm^2) te zijn. De lokatie van de AH en S neuronen binnen de ganglia bleek willekeurig te zijn. De NS-cellen lagen vaak in de buurt van een uitloper van een ganglia.

Op basis van de resultaten van het onderzoek beschreven in hoofdstuk 2 werd geconcludeerd dat het al of niet voorkomen van een schouder op de actiepotentiaal gebruikt kan worden om AH en S neuronen eenduidig te classificeren. De voordelen van deze wijze van classificeren, boven de bestaande classificatie schema's, zijn dat de cellen "on line" gekarakteriseerd kunnen worden en dat de indeling eenduidig is bij gebruik van slecht één criterium.

In **hoofdstuk 3** worden de effecten van CCK op de S neuronen beschreven, alsmede de betrokken CCK receptoren gekarakteriseerd. Toediening van CCK-8 aan deze neuronen veroorzaakte een langdurig verhoogde exciteerbaarheid van de meeste S neuronen via een direct effect op de neuronen. Deze verhoogde exciteerbaarheid was zichtbaar als de afgifte van spontane actiepotentialen of een verhoogde afgifte van actiepotentialen op depolariserende stroompulsen. De verhoogde excitatie was geassocieerd met een concentratie-afhankelijke (1-1000 nM CCK-8) depolarisatie en een toename in weerstand van de celmembraan.

Om na te gaan welke type receptor betrokken was bij de exciterende effecten van CCK op de S neuronen zijn selectieve antagonisten gebruikt voor de twee bekende typen CCK receptoren; de CCK_A en CCK_B receptor. Tot voor kort werd verondersteld dat CCK_A (alimentary) receptoren alleen perifeer voorkomen en de CCK_B (brain) receptoren alleen in het centraal zenuwstelsel. De laatste tijd komen er echter steeds meer aanwijzingen voor de aanwezigheid van CCK_A receptoren in het centrale zenuwstelsel en CCK_B receptoren in de periferie. Op grond hiervan zou verwacht mogen worden dat de CCK_A receptoren betrokken zijn bij de effecten van CCK op de cellen.

Experimenten met de selectieve CCK antagonisten L-364,718 (CCK_A receptor antagonist) en L-365,260 (CCK_B receptor antagonist) toonden aan dat beide CCK receptoren betrokken waren bij de neurale response op CCK-8. Drie groepen S neuronen konden onderscheiden worden, gebaseerd op de aanwezigheid van type CCK receptor. De neuronen bezaten ofwel de CCK_A receptor, ofwel de CCK_B receptor, ofwel beide typen receptoren. Deze resultaten toonden voor de eerste keer aan dat in het EZS behalve CCK_A ook CCK_B receptoren voorkomen. Het voorkomen van beide typen receptoren op individuele neuronen was tot nu toe alleen nog

aangetoond in het centrale zenuwstelsel.

Activatie van de verschillende CCK receptoren was altijd geassocieerd met een verhoging van de membraan weerstand. De effecten van CCK-8 geïnduceerd via de verschillende receptoren bleken echter verschillend te zijn. CCK effecten gemedieerd via de CCK_A receptor waren langduriger en gingen gepaard met afgifte van spontane actiepotentialen, in tegenstelling tot de effecten gemedieerd via de CCK_B receptor.

Samenvattend werd uit de resultaten geconcludeerd dat CCK een exciterend effect heeft op de meeste S neuronen gemedieerd door zowel CCK_A als CCK_B receptoren. Verder kwam uit het onderzoek naar voren dat een deel van de cellen of alleen de CCK_A receptor of alleen de CCK_B receptor bezat, terwijl een ander deel van de cellen beide receptoren bezat.

In **hoofdstuk 4** worden de effecten van CCK op de AH neuronen beschreven. Toediening van CCK-8 aan deze cellen veroorzaakte zowel exciterende als inhiberende effecten op deze cellen. In ongeveer de helft van de AH neuronen veroorzaakte toediening van CCK-8 een langdurige excitatie, geassocieerd met een onderdrukking van de na-hyperpolarisatie. In tegenstelling tot de effecten op de S neuronen, ging de door CCK-geïnduceerde excitatie nooit gepaard met de afgifte van spontane actiepotentialen. Toediening van niet-gesulfateerd CCK, CCK-8NS (een CCK_B agonist), aan deze cellen veroorzaakte een vergelijkbaar effect als CCK-8. De effecten van CCK-8 (1-1000 nM) en CCK-8NS (1-1000 nM) bleken concentratie-afhankelijk. Lage concentraties (<30 nM) veroorzaakten een excitatie gekenmerkt door een afname in weerstand. Onderzoek met selectieve receptor antagonisten (L-364,718 (250 nM), CCK_A; L-365,260 (250 nM), CCK_B) toonden aan dat deze afname gemedieerd werd door CCK_B receptoren. Hogere concentraties CCK-8 en CCK-8NS induceerden een additionele toename in membraan weerstand welke tot stand kwam via een effect op de CCK_A receptoren. De verschillende weerstand veranderingen geïnduceerd door CCK bleken dus receptor gebonden, en waren waarschijnlijk het gevolg van verschillen in betrokken ionkanalen.

In een klein deel van de AH cellen (11%) veroorzaakte toediening van CCK een inhibitie van de cel, gekenmerkt door een vergroting van de na-hyperpolarisatie. De inhiberende response ging gepaard met een afname in de membraan weerstand. Onderzoek met voornoemde CCK receptor antagonisten en CCK-8NS toonden aan dat deze response tot stand kwam via een effect van CCK op de CCK_B receptoren. Dit houdt in dat CCK, via een werking op de CCK_B receptoren, zowel een exciterend als een inhiberend effect tot gevolg kan hebben in AH neuronen. Het onderliggende

mechanisme is in beide gevallen een verlaging in membraan weerstand.

Samenvattend werd uit de resultaten van dit onderzoek geconcludeerd dat CCK overwegend een exciterend effect heeft op de AH neuronen gemedieerd door zowel de CCK_A als de CCK_B receptor. Tevens kon geconcludeerd worden dat de twee typen receptoren niet alleen een verschil in affiniteit voor CCK vertonen, maar dat de beide receptoren via verschillende ionkanalen het exciterend effect medieren.

Doel van het onderzoek beschreven in **hoofdstuk 5** was om te onderzoeken of CCK als neurotransmitter betrokken is bij langzaam synaptische responsen (sEPSPs). Hiertoe werden de effecten van gelijktijdige toediening van selectieve CCK_A en CCK_B receptor antagonisten op via elektrische stimulatie opgewekte sEPSPs onderzocht. Deze sEPSPs werden opgewekt door elektrische stimulatie van een uitloper van een ganglion. De sEPSPs werden gezien als een langdurige verhoogde excitatie van de neuronen, gepaard gaande met een depolarisatie van de cel en een weerstand verandering. Gelijktijdige toediening van de CCK receptor antagonisten L-364, 718 en L-365,260 (elk 250 nM) veroorzaakte een significante onderdrukking van de sEPSPs in een deel van de neuronen. Zowel de depolarisatie als de weerstandsverandering werden onderdrukt.

Op basis van de resultaten van dit onderzoek werd geconcludeerd dat neuronaal afgegeven CCK een rol speelt in de totstandkoming van langzame synaptische responsen in een deel van de neuronen. CCK voldoet nu aan de belangrijkste criteria voor een functie als neurotransmitter in het EZS: (1) CCK is aanwezig in neuronen; (2) CCK heeft een effect op de neuronen (dit proefschrift); (3) CCK komt vrij uit de neuronen na elektrische stimulatie van de zenuwvezels (dit proefschrift).

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Arma

Curriculum vitae

Irma Wilhelmina Maria Schutte werd op 23 november 1964 geboren in Bennekom, gemeente Ede. In 1983 behaalde zij het VWO diploma aan het Heldring College te Zetten. In hetzelfde jaar begon zij aan haar studie Zoötechniek aan de Landbouwuniversiteit te Wageningen. In augustus 1989 studeerde zij af met Veevoeding en Dierfysiologie als afstudeervakken. Na het afstuderen is zij gedurende acht maanden werkzaam geweest bij het Department of Animal Science, Oklahoma State University, Stillwater, USA. Vanaf december 1990 was zij vijf jaar werkzaam als Onderzoeker in Opleiding bij de vakgroep Fysiologie van Mens en Dier van de Landbouwuniversiteit te Wageningen, waar het in dit proefschrift beschreven werk werd verricht.