PHD THESIS

FUNCTIONAL „PURINERGIC” INNERVATION OF THE INTESTINE

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INTRODUCTION

In the beginning of the 20th century J.N. Langley discovered that the ganglionated nerve plexuses of the gastrointestinal tract (GIT) containing millions of neurons form an integrative nervous system that functions independently from the central nervous system. It became evident that the enteric nervous system (ENS) is the third part of the autonomic nervous system, besides the sympathic and parasympathic parts of it. The ENS regulates the movements, blood flow (vasomotor reflex), mucosal transport mechanisms, and modulates the immune and endocrine functions of the GIT with its several reflex pathways and nerves. The ENS contains almost the same amount of neurons as the spinal cord. There exists a lot of established and potential transmitters in the ENS (e.g. acetylcholine, nitric oxide (NO), adenosine-5'-triphosphate (ATP), vasoactive intestinal polypeptide (VIP), pituitary adenilate cyclase activating peptide (PACAP), calcitonin gene related peptide (CGRP), cholecystokinin octapeptide (CCK-8), carbon monoxide (CO), tachykinins, etc.). Several transmitters can be detected both in the neurons of the gut and in those of the central nervous system. The ENS is able to coordinate the intestinal movements independently from the central nervous system; gut functions seem not to be injured in the absence of the whole extrinsic nerve supply (this is not valid for the most proximal and the most distal part of the GIT).

Intrinsic neurons of the ENS form two main ganglionated nerve plexuses: plexus myentericus and submucosus. Nerve networks without ganglia can be found in all layers of the GIT wall. Thus, a complex and continuous neuronal network is present in the GIT. Intrinsic primary afferents are the first neurons of the enteric reflex pathways. They make synapses with interneurons, motoneurons, and they can activate other intrisic primary afferents too. Most of the enteric nerves are ascending or descending interneurons that give multiple neuronal connections between the sensory and effector neurons. Effector neurons can end on the circular or the longitudinal smooth muscle (motoneuron), on vascular smooth muscle cells (vasomotor neuron), on the mucosal glands (secretomotor neuron) and also on pacemaker cells. Motoneurons can be excitatory and inhibitory (causing, e.g., smooth muscle contraction or relaxation). Based on the phenomenon of cotransmission, several transmitters can be released at the same time from the activated neurons in the ENS. Although the ENS is able to work independently from the central nervous system, extrinsic nerves also play part in the neuronal regulation of the GIT. Sympathetic postganglionaric noradrenergic neurons innervate the sphincters where, acting on α_1 receptors, they evoke contraction producing a higher tone of these smooth muscle rings. Sympathetic postganglionic neurons innervate the arterioles of the intestine where, besides norepinephrine, ATP acts as a cotransmitter, producing vasoconstriction.

Extrinsic afferents are also involved in the innervation of the GIT. Sensory neurons of the spinal cord release transmitters in the periphery (also in the GIT) mediating a local efferent role in visceral organs. These sensory neurons can be activeted by capsaicin, the sensory stimulant drug of red pepper (paprika). Based on the modified Dale's principle it is probable that the same transmitter combination is released from the peripherial and from the central nerve endings. Thus transmitters released from the nerve endings of extrinsic afferent neurons are the same in the spinal cord and in the intestine.

Electrical stimulation of intramural nerves of an intestinal preparation in the presence of the muscarinic receptor antagonist atropine and the adrenergic neuron blocker guanethidine causes a non-adrenergic, non-cholinergic (NANC) response, which can be excitatory or inhibitory. Transmitters producing these responses are called NANC

neurotransmitters. After the blockade of muscarinic acetylcholine receptors there is still an excitatory component in the motor response, for which tachykinins (substance P, neurokinin A) are thought to be responsible. There is a dynamic research concentrating on the neuroeffector role of neurotesin, galanin, endothelins, bombesin-like peptides, „vasoactive intestinal contractor", cholecystokinin. „Nitrergic", „purinergic" and „peptidergic" neurons are responsible for the NANC relaxation. The most important enteric inhibitory neurotransmitter is NO, but there is evidence for the transmitter role of ATP, VIP, PACAP, CGRP, neuropeptide Y, pancreatic polipeptide and CO. Some of the possible transmitters (e.g. endothelins) can produce both excitatory and inhibitory response.

Our aim was to investigate the **„purinergic" innervation of the intestine.**

It is well know that ATP has a prominent role in the metabolism as intracellular energy store for living cells. It has also been known for a long time that it takes part in the synthesis of genetic material as a nucleic acid precursor. Szent-Györgyi and Drury described for the first time the extracellular effects of ATP in the cardivascular system. The term „purinergic" is created by Burnstock (Burnstock, 1972). Several articles confirmed that ATP, adenosine and other purine nucleotides and/or nucleosides play an important role as neurotransmitters, co-transmitters or neuromodulators in the regulation of the GIT. Besides of the peripheral effects of ATP, there is an intensive research for analyzing the role of ATP in the central nervous system too. ATP is co-transmitter in autonomic neurons, may play a role in nociception and in the mediation of visceral pain. In pathologic states and with aging more „purinergic" nerves are involved in regulatiory mechanisms. ATP and related compounds activate purinoceptors (purine-receptors). Purine-nucleotide selective P_2 purinoceptors include P_{2X} ligand gated cation-channels and P_{2Y} G-protein-linked receptors. The ATP analogues with greater metabolic stability, e.g. α,β-methyleneATP (α,β-meATP) are also used for the examination of purinergic neurotransmission. These ATP analogues have different receptor spectrum from ATP. There exist receptor-subtype selective agonists too, e.g. β,γ-methylene ATP (for the $P2X_1$ receptor), ADPβS (for the $P2Y_1$ receptor).

Several methods are available for identifying „purinergic" neurotransmission. We used three of them: 1. Desensitization with α,β-meATP 2. Inhibition with antagonists. For the identification of the involvement of purinergic nerves in a NANC response there is no need for a receptor-subtype-specific antagonist; drugs with specificity to purinoceptors or P_2 purinoceptors are needed only. The ATP released probably acts on several receptor subtypes at the same time if they are present on the target cell. 3. Inhibition with apamin. This polypeptide has an inhibitory effect on small-conductance K^+-channels, which mediate the main part of the relaxing effect of ATP on smooth muscle. It has recently been reported that smooth muscle inhibitory actions of other agents in animal gut preparations are also apamin-sensitive, e.g. relaxation evoked by various neuropeptides. ATP probably does not play a mediating role in an apamin-insensitive NANC relaxation, but an apamin-sensitive response is not certainly „purinergic".

It is known that ATP has three major roles in the ENS: 1. released from enteric motoneurons ATP acts as an inhibitory neurotransmitter on smooth muscle P_{2Y} receptors; 2. it is excitatory neurotransmitter acting between enteric interneurons or interneuron and motoneurons; 3. ATP acts as a sensory transmitter on the nerve endings of the intrinsic sensory neurons. It became evident that ATP has a mediating role both in excitatory and inhibitory responses of the GI smooth muscle; ATP is the major mediator of the apamine-sensitive inhibitory junction potential evoked by electrical stimulation. ATP and NO are cotransmitters in the ENS. Smits and Lefebvre showed

that ATP desensitization diminishes the NANC relxation on rat ileal strips. Our workgroup proved with the use of antagonists that ATP and NO together are responsible for NANC relaxation in the guinea pig taenia caeci and in rat plexus myentericus ileal strip preparation (Barthó et al., 1998; Benkó et al., 2006).
Roles of purine and pirimidine neurotransmitters are proven in many physiological and pathophysiological mechanisms. Due to plasticity of purinoceptor expression in certain disorders the „purinergic" component is significantly augmented in autonomic cotransmission. Drugs acting on „purinergic" systems are hot topic in laboratory research worldwide. Target diseases for drugs acting on „purinergic" signal transduction are: neurological disorders (neurodegeneratve diseases, epilepsy, stroke), inflammatory diseases (inflammatory bowel diseases), urological diseases, cancers, painful conditions, sensory organ disorders, immunological, cardiovascular diseases (Burnstock, 2006, 2007). Development of selective agonists, antagonists, P2 receptor expression modulators, inhibitors of ATP hydrolisis and transport mechanisms could provide new perspectives in the treatment of several diseases.

AIMS

Our in vitro experiments focused on the analysis of the mechanisms which regulate the movements of visceral organs of laboratory animals and the human intestine. Our aim was the identification and study of regulating roles of the sensory and other non-adrenergic, non-cholinergic (NANC) neurotransmitters. When assessing the mediating role of a given putative neurotransmitter, we concentrated on the criteria of „identity of action" and, first of all, on „identity of antagonism". Experiments were performed on preparations from laboratory animals and on human tissues (the latter in collaboration with the Department of Surgery). Our experiments belong to basic research, with possible future clinical relevance. Our results could help in the better understand of the function of the GIT, as well as in the identification of the pathomechanisms responsible for the motility disorders. Drugs that modulate adrenergic and cholinergic transmitter systems are widely used today. A deeper understanding of the NANC systems could help in the development of new drugs. Based on the Dale principle transmitter identification in the peripheral tissues, including visceral organs could have relevances for mechanisms in the spinal cord or in the brain sensory nuclei, thus the identification of NANC transmitter systems could provide new insights to other pathophysiological mechanisms (e.g. inflammation, nociception, pain).

Specific aims of the experiments:

1. • Our aim was to better understand the possible transmitter role of ATP acting between neurons or neurons and smooth muscle cells in the guinea-pig ileum. To this end we examined the multiple motor effects of exogenous ATP and α,β-meATP on the guinea-pig small intestinal longitudinal muscle.

• We made experiments to analyse the mechanisms responsible for these effects, and examined whether the P_2 purinoceptor antagonist PPADS is able to antagonize these motor effects.

• We made efforts to identify the purinoceptor subtypes mediating the effect of α,β-meATP. An attempt was made to quantify the degree of tachyphylaxis to α,β-meATP and the effect of tachyphylaxis on cholinergic contraction in the guinea-pig small intestine.

2. • On human ileal circular muscle the hypothesis was tested whether „purinergic" nerves play a mediating role in the NANC inhibitory response; to this end we examined the effect of the $P2Y_1$ antagonist MRS 2179 on the human ileum circular muscle.

3. We investigated the effect of tachyphylaxis to VIP or capsaicin on NANC inhibitory responses on the human sigmoid colon circular muscle, to assess whether VIP or sensory neuropeptides (or other sensory neurotransmitters) play a role in this response.

EXPERIMENTAL

I. Effects of ATP and α,β-methylene ATP in the guinea-pig small intestine

Introduction

In the enteric nervous system NO is responsible for the most part of the NANC responses. Besides NO, „purinergic" nerves may have a mediating role in these responses (see Burnstock, 1972, 1997). α,β-meATP is an analogue of ATP with a greater metabolic stability than the parent compound. Though its spectrum of agonist activity at various purinoceptors is somewhat different from that of ATP itself, α,β-meATP is one of the traditional drugs used for studying „purinergic" mechanisms in different preparations. In addition to stimulating some subtypes of P_{2X} purinoceptors, it can induce powerful tachyphylaxis on these receptors. Less data are available of the effect of purinoceptor antagonists on NANC and other responses. Based on the data of our workgroup, PPADS has a specific inhibitory effect on excitatory or inhibitory motor effects mediated by P_2 purinoceptors on different gastrointestinal preparations of the guinea-pig, which underline the neurotransmitter role of ATP or a related substance. Given the diversity of results concerning ATP in the guinea-pig small intestine, this study set out to determine the motor effects of exogenous ATP. We feel that, at this stage, the main question is not as to which types of purinoceptors mediate the effects of ATP, but it is to find a way to inhibit all of ATP effects. A broad-spectrum antagonism helps to decide the question whether purinergic nerves may or may not be involved in various nerve-mediated responses. Here we describe the complex effects of exogenous ATP on the longitudinal muscle of the guinea-pig small intestine, and we tried to analyse the possible mechanisms mediating these effects. We analysed the effect of *α,β-meATP tachyphylaxis on cholinergic contractions. In the present study, specificity testing of PPADS* was extended to a high concentration of this drug (300 μmol/l), using exogenous acetylcholine or histamine as spasmogens. Efforts have been made to quantify the degree of tachyphylaxis caused by *α,β-meATP and for getting more insight*

into the mechanism of the excitatory action of α,β-meATP in the guinea-pig ileum and see if the P_2 purinoceptor antagonist PPADS is able to antagonize the effect of this ATP analogue. In an attempt to better identify the subtypes of purinoceptor mediating the effect of α,β-meATP we have examined the sensitivity of this response to NF 279, a receptor antagonist acting at $P2X_{1,2,3}$ purinoceptors and to Brilliant blue G, a $P2X_{5,7}$ receptor antagonist with some affinity for $P2X_2$ and the human $P2X_4$ receptor.

Methods

Albino guinea-pigs were stunned by a blow to the occiput and bled out from carotid arteries. Whole segments of the ileum were set up as preparations and were suspensed in organ bath containing oxygenated Krebs-Henseleit solution at 37°C. Longitudinal movements were recorded isotonically, using lever transducers and bridge amplifiers. Nerves were activated by electrical fiel stimulation (EFS; 5 Hz, 5s). Contractions were expressed as % of the maximal longitudinal spasm at the end of the experiment.

Results

I. Motor effects of ATP in the small intestine of guinea-pig

The effects of ATP were studied with the non-selective P_1 purinoceptor antagonist theophylline in the organ bath. At lower concentrations (1-10 μmol/l) ATP caused a transient inhibition of the spontaneous movements of the preparations or a small relaxation. At higher concentrations (30 μmol/l - 1 mmol/l) ATP induced two types of contraction in part of the preparations: (a) A fast phasic contraction appeared in 28 out 80 preparations studied. Most of these responses also had a slower phase of contraction. (b) In some preparations, the phasic contraction could not be elicited in the concentration range of ATP tested, but a tonic contraction, peaking at sec. 10-30 was present. This response (and possibly also the slow phase of the response described above) probably represents the one first described by Moody and Burnstock, and Watt in 1982. The relaxant effect of ATP was studied in atropine- and guanethidine-treated, histamine precontracted ileum segments. ATP caused relaxation in all preparations. Threshold concentration of was ATP 0.3-0.5 μmol/l and the response was maximal at 10 μmol/l of ATP. A repeated administration of the same amount of ATP after the first one was ineffective with the phasic contraction and the relaxant response. It seems feasible that in those cases where the second administration was also effective (tonic contraction) it is possibly the ATP metabolism that causes the fading of the first response, while in the other case probably ATP-tachyphylaxis seems to play a role.

II. Sensitivity of the ATP-induced contractions to drugs

1. Phasic contraction

The phasic contraction due to ATP (300-500 μmol/l) was abolished by atropine and TTX, reduced by the ganglion blocker hexamethonium, but left uninfluenced by capsaicin tachyphylaxis. PPADS abolished the phasic contraction. α,β-meATP tachyphylaxis − surprisingly − had no effect on the phasic contraction.

2. Tonic contraction

The tonic contractile response to ATP was not diminished by atropine, TTX, hexamethonium or α,β-meATP tachyphylaxis, but was abolished by PPADS (30 μmol/l), and strongly inhibited by 3 μmol/l PPADS. ATP-induced tonic contractions were not inhibited by ω-conotoxin GVIA (N-type Ca^{2+}-channel blocker). Tonic contractions that followed an initial phasic one had the same pharmacological sensitivity towards atropine, hexamethonium, TTX, capsaicin, PPADS and α,β-meATP as did primary tonic contractions. Contractions evoked by ATP in the presence of atropine or TTX always had a tonic character. This response was strongly inhibited by PPADS.

3. Relaxation in response to ATP

ATP-induced relaxations were not influenced by TTX. Apamin (Ca^{2+}-dependent K^{+}-channel blocker) fully and irreversibily blocked relaxations in response to ATP (1 - 100 μmol/l). PPADS inhibited the relaxant response to ATP. The effect of PPADS was apparently surmountable through an elevation of the ATP concentration. A similar pattern of inhibitory effect was also obtained with reactive blue 2 (10 μmol/l). A mixture of the nitric oxide synthase inhibitor L-NOARG and ω-conotoxin GVIA failed to influence ileum relaxation due to ATP. Apamin and PPADS failed to influence histamine- or acetylcholine-evoked contractions.

III. Motor effects in response to α,β-meATP on guinea-pig ileum

α,β-meATP (1-30 μmol/l) caused concentration-dependent contractions in the ileum. Powerfull tachyphylaxis was present. In atropine-treated, histamine-precontracted preparations α,β-meATP failed to evoke any consistent relaxant effect in a concentration range of 1-30 μmol/l. The motor effect of α,β-meATP was fully blocked by the Na^{+}-channel blocker TTX or the broad-spectrum muscarinic receptor antagonist atropine. Hexamethonium failed to influence the contractile effect of α,β-meATP. The contractile effect of α,β-meATP (3-30 μmol/l) was strongly inhibited by PPADS. NF 279, a purinoceptor antagonist acting at $P2X_{1-3}$ receptors concentration-dependently reduced the contractile effect of α,β-meATP. Brilliant blue G failed to influence the effect of α,β-meATP.

IV. Effect of α,β-meAT tachyphylaxis on guinea-pig ileum

An attempt was made to quantify the degree of tachyphylaxis to α,β-meATP. It was found that even the smallest concentration tested (1 μmol/l) strongly reduced the effect of a further administration of the same amount or of 10 μmol/l α,β-meATP. Tachyphylaxis to α,β-meATP (1-10 μmol/l) was fully reversible within 20 min of washing. Neither the contractile effect nor tachyphylaxis to α,β-meATP was influenced by a combination of the adrenergic neuron blocking drug guanethidine, the broad-spectrum opioid receptor antagonist naloxone and the broad-spectrum NO synthase inhibitor L-NOARG.

V. Effect of α,β-meATP tachyphylaxis on nerve-mediated ileum contractions

α,β-meATP (1-10 μmol/l, administered for 20 min. without rinsing) caused a moderate inhibition of the (mostly cholinergic) response due to EFS. The effect reached its peak (approximately 30 % reduction) at 3 μmol/l of α,β-meATP and was not enhanced at higher contrentrations of the drug. The inhibitory effect of α,β-meATP (10 μmol/l) was not changed in the presence of naloxone, guanethidine and L-NOARG. Responses to nicotine were not significantly influenced by α,β-meATP tachyphylaxis.

Discussion

At least three types of motor response to ATP can be distinguished in the guinea-pig ileum on the basis of the present results, and none of them are identical with the response evoked by α,β-meATP. Optimum concentrations for the relaxation are clearly lower than those for the contractile responses. All responses observed faded away with time, however both tachyphylaxis and a fall in the actual drug concentration seem to play a role in the fading.

Sensitivity of the ATP-induced contractions to drugs

The tonic contraction, and possibly also the slower contraction following the phasic one are probably identical with the contraction described by Moody and Burnstock (1982), Watt (1982) and others. This statement is based first of all on the lack of atropine- and TTX-sensitivity of these types of contractions, which probably points to a postjunctional effect of ATP. In contrast to the tonic contraction, the fast (phasic) contraction with ATP seems to be fully mediated by cholinergic neurons of the gut wall. A reduction of ATP-evoked phasic contraction by hexamethonium might indicate that the purinoceptors mediating the phasic contraction are situated not only on enteric cholinergic motoneurons, but also presynaptically on neurons providing cholinergic nicotinic input to these neurons. We also tried to investigate the problem of activating nerve endings by ATP by including the N-type voltage-dependent Ca^{2+}-channel blocker ω-conotoxin GVIA. Neither the ATP-induced primary tonic contraction nor the relaxant effect of ATP was influenced by a relatively high concentration of ω-conotoxin GVIA. Even a combination of TTX and ω-conotoxin GVIA failed to reduce these ATP-induced responses. It would therefore seem that the tonic contractile and the relaxant effects of ATP are exerted via receptors on the smooth muscle. Relaxant responses to ATP were also resistant to TTX or L-NOARG, which makes it unlikely that NO of any source would play a role in this effect. Electrophysiological studies on myenteric neurons indicate fast excitatory potentials due to exogenous ATP, as well as „purinergic" fast potentials in response to electrical stimulation of presynaptic input (Barajas-Lopez et al., 1993, Galligan and Bertrand, 1994). These electrophysiological responses may have the same mechanism as the fast response seen in the current experiments. No attempt has been made for determining the exact type(s) of receptor mediating the effects of ATP in the ileum, because the availability of specific antagonists acting on P_2 purinoceptor subtypes is limited. It is probable that ATP can activate several types and subtypes of purinoceptor at the same time. All there motor responses to ATP were inhibited by PPADS at a concentration of 30 μmol/l or below. Concerning ATP-induced

relaxation, the inhibitory action of PPADS (30 μmol/l) seems to be surmountable. As to the contractile responses, concentration-response relationships could not be determined since the concentrations of ATP producing these responses were quite high anyway. In this case, however, lowering the concentration of PPADS tended to have a weaker inhibitory effects, yet the reduction of responses exceeded 50 % even with 3 μmol/l PPADS. Data of literature indicate EC_{50} values of PPADS in the micromolar range, in some types of receptors can be higher (see Benkó et al., 2007). With all this in mind, PPADS can probably be safely used for inhibiting P_2 purinoceptor-mediated responses. We now present evidence that not only α,β-meATP, but also ATP itself is able to excite cholinergic neurons of the myenteric plexus, in a PPADS-sensitive manner. This is in line with our previous observations that PPADS (Barthó et al., 1997) or α,β-meATP tachyphylaxis (present findings) moderately inhibit cholinergic contractions in response to EFS that may contribute to ATP release in the vicinity of enteric cholinergic motoneurons. An inhibitory effect of PPADS on the ATP-induced responses has also been taken as evidence for an effect on P_2 purinoceptors, not on P_1 receptors (the likelihood of this latter was also diminished by the use of theophylline). On the other hand, a significant inhibition of the ATP-induced relaxation by reactive blue 2 probably indicates an involvement of P_{2Y} purinoceptors in this type of response. These latter findings also imply that PPADS is likely to act, in a surmountable manner, on P_{2Y} purinoceptors, which has already been noted in early observations concerning this drug.

Effects of drugs on responses evoked by α,β-meATP

Most workers agree that α,β-meATP causes cholinergic contraction, i.e. activates excitatory motoneurons of the myenteric plexus in guinea-pig small intestine (see among others Kennedy and Humphrey, 1994).

α,β-meATP, at least in the concentration range studied in the present experiments, only contracts the longitudinal muscle of the ileum. Based on the present study it is probable that receptors mediating cholinergic contraction evoked by ATP and α,β-meATP are not the same, i.e. α,β-meATP at 10 μmol/l has no effect on phasic cholinergic contraction evoked by ATP. On the other hand administration of 10 μmol/l or even 1 μmol/l α,β-meATP strongly reduces the effect of a further administration of α,β-meATP. Moreover, the ganglionic blocker hexamethonium influences in a different way the cholinergic contraction evoked by ATP and α,β-meATP. As shown by the lack of inhibition by hexamethonium, preganglionic cholinergic nerve fibres probably do not play a significant role in α,β-meATP-induced contractile response. It is, however evident that the receptors involved are insensitive to relatively high concentrations of Brilliant blue G ($P2X_{5,7}$ receptor antagonist). They are sensitive to the broad-spectrum P_2 purinoceptor antagonist PPADS, and show moderate sensitivity to NF 279. The latter finding might indicate an involvement of $P2X_1$, $P2X_2$ or $P2X_3$ receptors.

The sensory stimulant capsaicin excites peripheral endings/varicosites of primary afferent neurons within the gut wall and transmitters released from capsaicin-sensitive afferents activate cholinergic neurones of the myenteric plexus (see Barthó et al. 2004). It is possible to block the function of capsaicin-sensitive afferents by a high concentration of capsaicin. The lack of inhibitory effect of capsaicin pretreatment on the ATP-induced phasic contraction practically excludes the involvement of capsaicin-sensitive neurons in this response. The contractile effect of α,β-meATP is also insensitive to capsaicin tachyphylaxis, as reported earlier (Barthó et al., 1999).

Effect of α,β-meATP tachyphylaxis on EFS- and nicotine-evoked responses

Insensitivity of nicotine-evoked cholinergic contractions towards α,β-meATP tachyphylaxis seems to reflect an interesting dichotomy of electrically versus drug-induced cholinergic contractions. This findings also speaks against any major postjunctional inhibitory effect of α,β-meATP tachyphylaxis. It should be noted that, while electrical field stimulation-induced cholinergic contractions are moderately inhibited by PPADS (Barthó et. al., 1997), cholinergic contractions in response to cholecystokinin octapeptide or the tachykinin NK_3 receptor agonist senktide are not influenced by this drug (Barthó et al., 2000). The mechanisms that make electrical activation of cholinergic neurons of the myenteric plexus more sensitive to P_2 purinoceptor inhibition remain to be elucidated.

Summary

It is concluded that, in addition to its direct contractile action in the guinea-pig ileum, ATP can activate (partly preganglionic) cholinergic neurons, an effect whose mechanism is largely different from that of the cholinergic contraction induced by α,β-meATP. ATP also causes relaxation by a direct, probably P_{2Y} receptor-mediated effect on the smooth muscle. All motor effects of ATP are inhibited by the antagonist PPADS. The cholinergic response to ATP and its inhibition by PPADS may underlie the reported partial inhibition by P2 purinoceptors of the cholinergic contraction of the guinea-pig ileum evoked by electrical field stimulation or stimulation of the mucosa.

α,β-meATP causes contraction in the guinea-pig ileum by stimulating PPADS-sensitive P_2 purinoceptors of cholinergic motoneurons of the myenteric plexus. Tachyphylaxis to α,β-meATP selectively reduces the effect of electrical activation of such neurons, which indicates an involvement of P_{2X} purinoceptors in excitatory neurotransmission in this organ.

II. „Purinergic" nerves mediate the non-nitrergic NANC relaxation of the human ileum in response to electrical field stimulation

Introduction

Functional innervation of the human small intestine is less well understood than that of experimental animals, especially the guinea-pig. In the human intestine, there is little doubt that NO plays an important role as a neurotransmitter in the NANC relaxation (see Burleigh, 1992; Maggi et al., 1991; Tam and Hillier, 1992). By contrast, no functional data are available to directly prove a participation of „purinergic" nerves in the response, though an apamin-sensitive mechanism has been described (Boeckxstaens et al., 1993). Recently it has become clear that apamin inhibits relaxation in response to other mediators as well. In the present study, the hypothesis was tested that P2 purinoceptors may play a role in the „non-nitrergic" component of the NANC relaxation in the human ileum. To this end, we examined the effects of the two P2 purinoceptor antagonists, PPADS and suramin, on the field stimulation-induced NANC relaxation in ileal strips pretreated with the NO synthase blocker L-NOARG.
A new and specific $P2Y_1$ receptor antagonist, MRS 2179 (Boyer et al., 1996) is frequently used for the investigation of the NANC relaxation of the intestine. The „non-nitrergic" NANC relaxation of the human sigmoid colon that is inibited in our experiments by the combination of PPADS and suramin (Benkó et al., 2007) was also inhibited with this antagonist (Gallego et al., 2006). It seems feasible that this $P2Y_1$ antagonis given as single drug is a more useful tool for identifying „purinergic" inhibitory mechanisms than previously-used drugs. We therefore analyzed the effect of MRS 2179 on the „purinergic" NANC relaxation of the human ileal circular muscle. This was preceded by a study on the specificity of this drug.

Methods

Human ileal tissue was obtained from gut segments surgically removed from carcinoma patients. The marginal, macroscopically intact zone was used. Circularly-oriented strips were prepared and freed of mucosa, and studied in conventional organ bath experiments, using oxygenated Krebs-Henseleit solution. Movements were recorded isotonically. Nerves were activated by electrical field stimulation (EFS). NANC conditions were maintained by atropine and the adrenergic blocking drug guanethidine. In most experiments L-NOARG (100 μmol/l) was also administered at the beginning of the experiment. The NANC relaxation was abolished by tetrodotoxin (1 μmol/l), an inhibitor of neuronal voltage-dependent Na^+ channels. Preparations were submaximally precontracted with neurokinin A, then EFS or a relaxing agent was administered (200 μmol/l ATP, 3-10 μmol/l isoprenaline). Responses are expressed as % of the maximal relaxation due to isoprenaline.

Results

All results have been obtained on precontracted preparations. Electrical field stimulation (1 or 10 Hz for 20s) elicited NANC relaxation that was significantly inhibited, but not abolished by L-NOARG (100 μmol/l).

In the presence of L-NOARG, the P_2 purinoceptor antagonists PPADS (50 μmol/l) or suramin (100 μmol/l) significantly inhibited the NANC relaxation at both frequencies of stimulation. A combination of the two antagonists caused a strong, approximately 70 % inhibition at 1 Hz, but only an approximately 35 % inhibition at 10 Hz.
Exogenous ATP (200 μmol/l) caused relaxations roughly of the same amplitude as electrical stimulation. PPADS and suramin reduced these responses by approximately 70 %.

The NANC relaxation of the human ileal circular muscles is inhibited by MRS 2179 , a P2Y purinoceptor antagonist

On preparations precontracted with neurokinin A MRS 2179 (3 or 10 μmol/l) significantly inhibited the „non-nitrergic" NANC inhibitory effect of electrical field stimulation at both 1 and 10 Hz. 3 μmol/l MRS 2179 caused approximately half-maximal reduction and 10 μmol/l MRS 2179 produced nearly full inhibition. Apamin (3 μmol/l) reduced by more than half the NANC relaxation at both 1 and 10 Hz.
Relaxation evoked by exogenic ATP was reduced significantly by 3 and nearly abolished by 10 μmol/l MRS 2179.
MRS 2179 (10 μmol/l) did not influence either isoprenaline-induced moderate relaxations or the contractile effect of neurokinin A.

Discussion

This study proved pharmacological evidence for first time for an involvement of P_2 purinoceptor-mediated „purinergic" mechanisms in the „non-nitrergic" NANC relaxation of the human small intestinal circular muscle.
NO clearly mediates part of the NANC responses, as demonstrated by two groups of investigators also for the circular muscle of the human small intestine (Maggi et al., 1991; Murr et al., 1999). In our experiments we confirmed the participation of NO in the NANC relaxation in the small intestinal circular muscle.
Our experiments confirmed that the antagonist MRS 2179, given as single drug is a useful tool for studying „purinergic" involvement in the human GI tract. Concentrations tested in the present study, 3-10 μmol/l MRS 2179 effectively inhibited the NANC relaxation, and significantly reduced the relaxating effect of the „purinergic" neurotransmitter ATP. Whether this concentration of MRS 2179 specifically inhibits $P2Y_1$ receptors or also acts on other types of purinoceptor has not been tested in the present study. Data from literature show specific effect of this drug to $P2Y_1$ receptors (see von Kügelgen and Wetter, 2000).
As conclusion, „purinergic" mechanisms, sensitive to the P2Y receptor antagonist MRS 2179 play an important role in the NANC relaxation of the human ileal circular muscle.

III. Effect of capsaicin and VIP tachyphylaxis on the NANC relaxation of the human colon

Introduction

There is three main candidates of neurotransmitters involved in the NANC relaxation of the intestine: NO, ATP or a related „purinergic" transmitter and VIP (or VIP/PACAP). NO and ATP were dealt with in the previous chapters.
A possible participation of VIP in the NANC relaxation of preparations from different parts of the gastrointestinal tract has been proposed (see Dockray, 1994; Lecci et al, 2002; Said and Rattan, 2004 for reviews).
Capsaicin is the pungent substance of paprika (red pepper, Capsicum annuum). It stimulates a specific, ion channel bound receptor (TRPV1) found on the sensory nerve endings (Caterina and Julius, 2001). Transmitters released from the capsaicin-sensitive sensory neurons of visceral organs are probably also released from the nerve endings of the sensory neurons in the central nervous system (in the dorsal horn of the spinal cord), thus may play a role in the nociception. Capsaicin is a valuable tool for sensory neurotransmitter indentification (see Barthó et al, 2004). Capsaicin desensitisation is widely used in isolated organ experiments because it produces a functional impairment of capsaicin-sensitive neurons.
GI preparations from different species show various responses to capsaicin (Barthó et al., 2004). Capsaicin has a TTX resistant inhibitory effect of the human intestine (Maggi et al., 1988, 1990a,b). Voltage-dependent fast sodium channels are not necessary for the sensory neurotransmitter releasing effect of capsaicin on sensory nerve endings, therefore most of the responses to capsaicin are resistant to TTX (Maggi, 1995). In the nineties, immuno-neutralizations and neurochemical experiments provided some evidence for the role of VIP or a related peptide in the effect of capsaicin. However CGRP – which mediates the inhibitory effect of capsaicin in animal preparations – plays no role in human preparations (Maggi et al., 1989, 1990a,b). Our laboratory showed for the first time that NO is an important mediator of the capsaicin-induced relaxation in human sigmoid colon (Barthó et al., 2002). Later we proved in several different GI preparations (human ileum, appendix, mouse colon) the role of NO in the capsaicin induced relaxation (Benkó et al., 2005).

Specific aims of the experiments:

1. The current experiments were designed to study the relaxing effect of exogenous VIP on human colon and to see the possible role of endogenous VIP in the „non-nitrergic" NANC relaxation of the human sigmoid colon circular muscle. In the absence of specific VIP antagonists, we used VIP tachyphylaxis to answer this question.

2. We studied whether capsaicin sensitive afferent neurons play a role in the NANC relaxation of human sigmoid colon circular muscle.

Methods and materials

Human sigmoid colon tissue was obtained from gut segments surgically removed from rectal carcinoma patients. The marginal, macroscopically intact zone was used. Circularly-oriented strips approximately 2 cm in length and 2 mm in width were prepared and freed of mucosa. Methods were the same as described above with the following differences: the relaxing effect of VIP (0.1 μmol/l) or capsaicin (0.3 μmol/l) were studied in the presence of guanetidine (3 μmol/l) on preparations precontracted with acetylchloline (1 μmol/l). To study the effect of VIP tachyphylaxis, 1 μmol/l VIP was administered for 60 min into the organ bath, then the relaxing effect of capsaicin (0.3 μmol/l) or VIP (0.1 μmol/l) were studied. In other types of experiments nerves were activated with electrical field stimulation in the presence of atropine (1 μmol/l) and guanethidine (3 μmol/l) for obtaining NANC conditions. After 1 hour incubation period the preparations were submaximally precontracted with histamine (5 μmol/l) then EFS (1 or 10 Hz) or isoprenaline were used.

Results

1. Effect of VIP tachyphylaxis on EFS evoked NANC relaxation and on capsaicin-sensitive inhibitory response

The half-maximal relaxating effect of VIP (0.1 μmol/l) was reproducible (n=8). High concentration of VIP (1 μmol/l) caused maximal relaxation, that faded away during the long incubation period. After one hour incubation the contractile effect of acetylcholine was the same as before the administration of VIP.
VIP tachyphylaxis (1 μmol/l for 60 min) strongly inhibited the relaxant effect of exogenous VIP (0.1 μmol/l), but failed to reduce the „non-nitrergic" TTX-resistant NANC response (1 Hz or 10 Hz) or the relaxation in response to isoprenaline (0.1 μmol/l).
VIP tachyphylaxis failed to inhibit the relaxant effect of capsaicin (0.3 μmol/l), which shows, on one hand, the specificity of this method, and on the other hand that VIP does not play an important role in the effect of the sensory stimulant capsaicin.

2. Effect of capsaicin tachyphylaxis on EFS evoked NANC relaxation

Relaxations to 1 or 10 Hz electrical field stimulation were not reduced by previous capsaicin treatment (10 μmol/l for 10 minutes, followed by a 40-min washout period). NANC relaxations were fully inhibited by TTX (1 μmol/l) (n=6).

Discussion

For assessing a possible role of VIP or PACAP we used VIP tachyphylaxis, because we have been unable to reliably inhibit the relaxant effect of exogenous VIP with the receptor antagonists tested. VIP tachyphylaxis failed to inhibit the effect of capsaicin or „non-nitrergic" electrical field stimulation. In fact we found some enhancement, whose mechanism still remains unclear. An endogenous VIP-like inhibitory transmitter probably plays no role in the NANC relaxation or in the relaxing effect of capsaicin. The possible role of VIP in NANC inhibitory responses was based on animal experiments (Grider et al., 1985). Evidence for the role of VIP in the capsaicin-induced

relaxation was published by Maggi and his collegues on the basis of immuno-neutralization and release experiments. On the other hand, they found no evidence for the role of the sensory neuropeptide CGRP in this response. In an attempt to get closer to the mechanism of the NANC inhibition we used capsaicin tachyphylaxis for inducing a functional blockade of the capsaicin-sensitive neurons. Capsaicin pretreatment failed to inhibit the relaxant effect of nerve stimulation.
In conclusion, no evidence has been found for an involvement of capsaicin-sensitive neurons in the „non-nitrergic" NANC relaxation in response to electrical field stimulation in the human colonic circular muscle, thus this response is mediated by intrinsic nerves.

SUMMARY OF THE NEW RESULTS

Results related to the potential neurotransmitter ATP and/or related substances:

- At least three types of motor response to ATP can be distinguished in the guinea-pig ileum on the basis of the present results. It seems that the tonic contractile and the relaxant effects of ATP are exerted via receptors on the smooth muscle. NO is not involved in the latter response.

- We provided evidence that not only α,β-meATP, but also the natural transmitter ATP is able to activate cholinergic neurons of the myenteric plexus in a PPADS-sensitive manner, probably with the stimulation of P_2 purinoceptors. It seems probable that the cholinergic excitatory responses evoked by ATP and α,β-meATP are mediated by different receptors.

- α,β-meATP tachyphylaxis selectively reduces the contraction evoked by electrical activation of cholinergic motoneurons, which indicates that P_{2X} purinoceptors may play part in the excitatory neurotransmission of the intestine.

- We provided pharmacological evidence for first time for an involvement of P_2 purinoceptor-mediated „purinergic" mechanisms in the „non-nitrergic" NANC relaxation of the human small intestine circular muscle. It is concluded that „nitrergic" and „purinergic" nerves play a mediating role in the NANC inhibitory effect due to electrical field stimulation in human ileum.

- Our results showed that the P_{2Y} purinoceptor antagonist MRS 2179, given as single drug, is a useful tool for studying „purinergic" involvement in the motor responses of the human ileum.

Results related to the potential neurotransmitter VIP:

- We have demonstrated that neither capsaicin-sensiteve neurons nor VIP participate in the „non-nitrergic" NANC relaxation in response to electrical field stimulation in the human colonic circular muscle. We did not find evidence for participation of VIP in the inhibitory response evoked by the sensory stimulant capsaicin, thus we conclude that VIP probably does not play a role as a sensory neurotransmitter.

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