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Evidence for the involvement of ATP, but not of VIP/PACAP or nitric oxide, in the excitatory effect of capsaicin in the small intestine

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Abstract

The contractile effect of capsaicin in the guinea-pig small intestine involves an activation of enteric cholinergic neurons. Our present data show that the P_2 purinoceptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM) significantly reduces the contractile response to capsaicin (2 μM) in the presence, but not in the absence, of the tachykinin receptor antagonists [O-Pro[9], (Spiro-γ-lactam)Leu[10], Trp[11]]physalaemin (1–11) (GR 82334; 3 μM) and (*S*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidine-4-yl)-*N*-methylacetamide (SR 142801; 100 nM) (for blocking tachykinin NK_1 and NK_3 receptors, respectively). PPADS (30 μM) fails to influence submaximal cholinergic contractions evoked by cholecystokinin octapeptide (CCK-8; 2–3 nM) or senktide (1 nM), or the direct smooth muscle-contracting effect of histamine (100–200 nM). A higher concentration (300 μM) of PPADS is also without effect against the stimulatory action of cholecystokinin octapeptide. This means that PPADS can probably be safely used as a purinoceptor antagonist in intestinal preparations. The putative pituitary adenylate cyclase activating peptide (PACAP) receptor antagonist PACAP-(6–38) (3 μM) significantly reduces the contractile effect of PACAP-(1–38) (10 nM) and abolishes that of vasoactive intestinal polypeptide (VIP; 10 nM). PACAP-(6–38) (3 μM) fails to influence the effect of capsaicin (2 μM) both in the absence and in the presence of tachykinin receptor antagonists. The nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine (L-NOARG; 100 μM) also fails to inhibit the capsaicin-induced motor response. We conclude that an endogenous ligand of PPADS-sensitive P_2 purinoceptors (possibly ATP), but not a VIP/PACAP-like peptide or NO, is involved in the nontachykininergic activation of cholinergic neurons in the course of the capsaicin-induced contraction.

Keywords: Capsaicin; Myenteric neuron; PPADS (Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid); VIP (Vasoactive intestinal polypeptide); PACAP (Pituitary adenylate cyclase activating peptide); NO (Nitric oxide); Small intestine

1. Introduction

Release of biologically active substances from capsaicin-sensitive primary afferent nerve endings evokes ''local efferent'' tissue responses (see Maggi, 1995; Szolcsányi, 1996 for reviews). It has been shown that the sensory neuron stimulant capsaicin excites sensory nerves within the gut wall, which activate neurons of the myenteric plexus by releasing tachykinins and some unknown transmitters (Barthó and Szolcsányi, 1978; Barthó et al, 1999a). A full understanding of this process would help identify sensory neurotransmitters that may also play a role in the central part of primary afferents. A supra-additive interplay of endogenous stimulants of tachykinin NK_1 and NK_3 receptors partly mediates the activation of myenteric neurons in the guinea-pig small intestine, whereas in the guinea-pig oesophagus tachykinin NK_1, NK_2, and NK_3 receptors seem to participate (Barthó et al., 1999a). However, in these preparations, a substantial component of the capsaicin-induced motor response is resistant to a combination of tachykinin NK_1, NK_2, and NK_3 receptor antagonists.

ATP is known to be present in and released from primary afferents (Holton, 1959; for reviews see, among others, Kennedy and Leff, 1995; Burnstock and Wood,

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1996; Barthó et al., 1999b) and is able to induce fast excitatory potentials/currents in myenteric neurons (LePard et al., 1997; see also Barthó et al., 1999b). Thus, it can be assumed that ATP of sensory neuronal origin could take part in the excitation of myenteric neurons. In fact, a pilot study showed that the P_2 purinoceptor antagonist suramin reduces the capsaicin-evoked motor response in the guinea-pig small intestine (Barthó et al., 1999b).

There are data to indicate that pituitary adenylate cyclase activating peptide (PACAP) and vasoactive intestinal polypeptide (VIP) are sensory neuropeptides (Jancsó et al., 1981; Moller et al., 1993; see Holzer, 1991; Sundler et al., 1996). These peptides relax the smooth muscle of many gastrointestinal preparations (see Dockray, 1994), but can also excite myenteric neurons (Williams and North, 1979; Cohen and Landry, 1980; Katsoulis et al., 1993; Rattan and Chakder, 1997; Pang and Kline, 1998; Heinemann and Holzer, 1999). The same is true for nitric oxide (NO) (see Barthó and Lefebvre, 1994, 1995; Rand and Li, 1995; Hebeiss and Kilbinger, 1996). NO may also be released from capsaicin-sensitive nerves (Aimi et al., 1991; Szikszay et al., 1998). Thus, the participation of VIP and PACAP, as well as NO in the neuronal excitation, in the course of the action of capsaicin should be considered. We have preliminary data to show an inhibition of the contractile response to capsaicin by a tachyphylaxis to VIP or PACAP-(1–27) (Barthó and Maggi, unpublished observations).

The aim of the present study was to investigate the possible involvement of ATP in the cholinergic motor response of the guinea-pig isolated small intestine to capsaicin. For this purpose, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (Lambrecht et al., 1992), a purinoceptor antagonist active on a number of P_2 purinoceptors was used. This substance may be somewhat more potent and specific than suramin as a purinoceptor antagonist (Humphrey et al., 1995). In our own experiments, PPADS (30 μM) fully antagonized the contractile effect of α,β-methylene ATP in the guinea-pig ileum, an effect stronger than that of suramin (100 μM; Barthó et al., 1997). It also turned out in preliminary experiments that quite high concentrations of PPADS are devoid of any detectable nonspecific effect, unrelated to purinoceptors, in the ileum (see Results).

We also tested the effect of PACAP-(6–38), a putative PACAP receptor antagonist (see Harmar et al., 1998), as well as the NO synthase blocker N^G-nitro-L-arginine on the motor response to capsaicin.

2. Materials and methods

2.1. Preparation

Standard isolated organ methods were used. Briefly, whole segments (approximately 2 cm long) of preterminal ileum from guinea-pigs weighing 350–460 g were suspended in organ baths containing Krebs solution of 37°C temperature and oxygenated by bubbling a mixture of 95% O_2 and 5% CO_2. Longitudinal movements of the segments were recorded isotonically, with a load of 5.5 mN (0.55 g). Experiments started after 60 min of equilibration. The preparations were exposed to acetylcholine (10 μM) at the beginning of the experiment. This was followed by another 30 min of rest.

In a separate set of experiments, ileum segments were pretreated with atropine (1 μM) and submaximally precontracted with histamine (0.1–0.2 μM). As soon as a stable baseline was obtained, PACAP-(1–38) or VIP was added for 3 min for studying their relaxant effect, then the bathing fluid was changed. This treatment was performed in cycles of 45 min.

2.2. Drugs

The following drugs were used: capsaicin, N^G-nitro-L-arginine (L-NOARG) (Sigma); cholecystokinin octapeptide (Bachem); GR 82334 or [O-Pro^9, (Spiro-γ-lactam)Leu^{10}, Trp^{11}]physalaemin (1–11) (Neosystem), SR 142801 or (*S*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidine-4-yl)-*N*-methylacetamide (Sanofi); pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (RBI); senktide or (Suc-Asp-Phe-Mephe-Gly-Leu-Met-NH_2), substance P, vasoactive intestinal polypeptide (VIP) (Peninsula). Pituitary adenylate cyclase activating peptide, as PACAP-(1–38), as well as the receptor antagonist fragment PACAP-(6–38) were synthesized in the Department of Medicinal Chemistry, University of Szeged Medical School or purchased from Bachem.

Solvents and stock solutions of capsaicin and tachykinin receptor antagonists have been described elsewhere (Barthó et al., 1999a). PPADS was dissolved in isotonic saline to give a stock solution of 100 mM; it was administered from a working dilution of 30 mM (also in saline), except when concentrations higher than 30 μM were used. Stock solutions of CCK-8 (10 μM), PACAP and its fragments, VIP (100–300 μM) and L-NOARG (10 mM) were prepared in isotonic saline. The solvents of drugs used in this study were without any effect on the preparations. Contact times for the drugs were as follows: 20 min for PPADS, L-NOARG, PACAP-(6–38) and GR 82334, and 60 min for SR 142801. Capsaicin (2 μM) was administered only once to each preparation for 3 min.

2.3. Statistics

Ileum contractions are expressed as percentage of the maximal longitudinal spasm due to acetylcholine (10 μM). Relaxations of the histamine-precontracted ileum are ex-

pressed as percentage of the prehistamine baseline. Data are presented as mean ± S.E.M. For statistical comparison of several unrelated samples (such as the effects of capsaicin with various pretreatments) the Kruskal–Wallis test was used. Wilcoxon's signed rank test was used for comparing a pair of related samples. Number of experiments (n) refers to the number of experimental animals used.

3. Results

Capsaicin (2 μM) induced half-maximal contractions in the ileum. These responses were significantly reduced by a combination of receptor antagonists acting at tachykinin NK_1 (GR 82334; 3 μM) and NK_3 receptors (SR 142801; 100 nM) (Fig. 1, Table 2). PPADS (30 μM) failed to inhibit the excitatory response to capsaicin in untreated preparations but considerably reduced it in preparations pretreated with GR 82334 plus SR 142801 (Fig. 1).

PPADS (30 μM) did not modify submaximal cholinergic contractions evoked by cholecystokinin octapeptide (2–3 nM); responses before the administration of PPADS reached 35.0 ± 4.7% of the maximal response to acetylcholine, while in the presence of PPADS they amounted to 34.4 ± 4.5% acetylcholine (10 μM; $n = 5$). Similar results have been obtained with CCK-8 versus PPADS if GR 82334 (3 μM) plus SR 142801 (100 nM) were present in the organ bath ($n = 4$, data not shown). A 10-fold higher concentration of PPADS (300 μM) was also ineffective against the excitatory response to CCK-8 (32.4 ± 2.3 and 31.2 ± 0.7% acetylcholine (10 μM) contraction without and with PPADS, respectively; $n = 5$). Cholinergic contractions with another agonist, senktide (a stimulant of tachykinin NK_3 receptors) also remained uninfluenced by PPADS (30 μM); responses with senktide (2–3 nM) reached 46.7 ± 4.6% before and 46.2 ± 4.2% in the presence of PPADS ($n = 6$). PPADS (30 μM) also failed to affect half-maximal contractions evoked by histamine (100–200 nM; 50.3 ± 3.4% contraction before and 50.9 ± 3.4% in the presence of PPADS; $n = 9$).

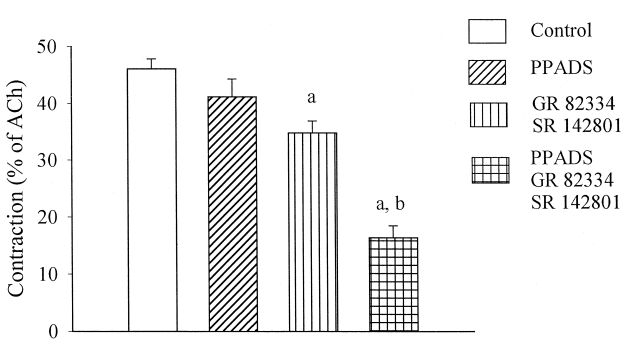

Fig. 1. Contractions of the guinea-pig ileum due to capsaicin (2 μM), expressed as percentage of the maximal longitudinal spasm evoked by acetylcholine (ACh; 10 μM). Concentrations of drugs were as follows, PPADS, 30 μM; GR 82334, 3 μM; SR 142801, 100 nM. Mean ± S.E.M. of 16–24 animals. Significant differences are shown above the columns; [a]$P < 0.001$ vs. control, [b]$P < 0.001$ vs. GR 82334 plus SR 142801. Contact times of drugs were as follows, 20 min for PPADS and GR 82334, 60 min for SR 142801.

Table 1
Antagonism of the contractile effect of PACAP-(1–38) and VIP on the guinea-pig ileum by PACAP-(6–38)
Contractions of the guinea-pig ileum evoked by PACAP-(1–38) or VIP (10 nM each) before and in the presence of the PACAP receptor antagonist PACAP-(6–38). Responses are expressed as percentage of the maximal longitudinal spasm with acetylcholine (10 μM). Mean ± S.E.M. are given. Statistically significant differences. Number of animals (n) is given at the top. Contact times of drugs were as follows: 3 min for PACAP-(1–38) and VIP and 20 min for PACAP-(6–38).

Pretreatment	Percentage contraction	n
PACAP-(1–38) (10 nM)		
–	26.3 ± 2.4	6
PACAP-(6–38) (3 μM)	16.0 ± 3.5[a]	
VIP (10 nM)		
–	24.1 ± 5.8	5
PACAP-(6–38) (3 μM)	ϕ[a]	

[a]$P < 0.05$ (Wilcoxon's signed rank test).

The putative PACAP receptor antagonist PACAP-(6–38), in a concentration of 3 μM significantly inhibited the contractile effect of PACAP-(1–38) (10 nM), although the reduction only reached 35% (Table 1). The contractile action of VIP (10 nM) was abolished by PACAP-(6–38) (3 μM; Table 1). The motor response to capsaicin (2 μM) was not altered by PACAP-(6–38) (3 μM; Table 2), either in the absence or in the presence of the tachykinin receptor antagonists GR 82334 and SR 142801. Likewise, L-

Table 2
Effect of PACAP-(6–38), L-NOARG and the tachykinin receptor antagonists GR 82334 and SR 142801 on the motor response of the ileum with capsaicin
Ileum contractions evoked by capsaicin (2 μM) alone or in the presence of various drugs and their combinations. Independent groups of observations. Kruskal–Wallis test was used for assessing statistically significant differences. Number of animals (n) is shown for each group. Contact time for L-NOARG was 20 min; those for the other drugs have been given with Fig. 1 and Table 1.

Pretreatment	Percentage contraction	n
Capsaicin (2 μM)		
–	50.6 ± 1.6	9
PACAP-(6–38) (3 μM)	52.3 ± 2.2	8
L-NOARG (100 μM)	57.9 ± 2.5	8
GR 82334 (3 μM) + SR 142801 (100 nM)	39.6 ± 4.4[a]	9
PACAP-(6–38) (3 μM) + GR 82334 (3 μM) + SR 142801 (100 nM)	37.5 ± 2.8[b]	8
L-NOARG (100 μM) + GR 82334 (3 μM) + SR 142801 (100 nM)	37.7 ± 4.8[a]	8

[a]$P < 0.05$ compared with the control group (capsaicin alone).
[b]$P < 0.01$ compared with the control group (capsaicin alone).

NOARG (100 μM) failed to significantly change the contractile effect of capsaicin (Table 2), although a slight tendency to enhancement seems to be present.

In a separate set of experiments performed on atropine-treated, histamine-precontracted ileum segments PACAP-(1–38) (10 nM) induced reproducible relaxation that was strongly inhibited by PACAP-(6–38) (3 μM); control responses reached 26.4 ± 2.1%, while those in the presence of the antagonist amounted to 3.9 ± 2.4% (the prehistamine baseline being taken as 100%) ($n = 5$; $P < 0.05$). Similar experiments with VIP (10–100 nM) yielded variable control responses (lack of relaxation in part of the preparations), hence the effect of PACAP-(6–38) was not tested.

4. Discussion

These data provide evidence that ATP or a similar endogenous ligand of PPADS-sensitive purinoceptors is involved in the excitatory effect of capsaicin in the small intestine. Under the circumstances of the present study, the response to capsaicin is abolished by atropine or tetrodotoxin (Barthó et al., 1999a). This probably indicates that the site of action of the ATP released is on the myenteric cholinergic neurons. The source of ATP might be capsaicin-sensitive afferent nerve endings, although we have no direct evidence for this. It was only the tachykinin receptor antagonist-resistant component of the capsaicin-induced contractile response that was significantly reduced by PPADS. We propose that, in the absence of the tachykinin receptor antagonists, the purinoceptor-mediated cholinergic response is masked by the tachykinin-mediated one.

PPADS has been reported to reduce, hence ATP to participate in, the cholinergic contractile response of the ileum evoked by electrical field stimulation (Barthó et al., 1997). In the present study, three types of drug-induced cholinergic contraction remained uninfluenced by PPADS, namely the response to capsaicin in the absence of tachykinin receptor antagonists, the CCK-8-and the senktide-induced contraction, which are also mediated by cholinergic neurons (Vizi et al., 1973; Laufer et al., 1985). It would seem that an activation of intramural nerves by electrical impulses liberates sufficient amounts of ATP for a positive modulation of cholinergic transmission, and this is not the case with drug-induced cholinergic responses.

PPADS (30 μM) has no smooth muscle-relaxing activity, as tested against acetylcholine and noncholinergic nerve stimulation (Barthó et al., 1997) or histamine (present study). Even a 10-fold higher concentration of PPADS (300 μM) is free of such an effect against histamine and noncholinergic nerve stimulation (L. Barthó, data in preparation), as well as CCK-8 (present study). Ineffectivity of PPADS against nerve-mediated responses also rules out a series of other effects, like local anaesthetic, adrenergic alpha, opioid, P_1 purinoceptor agonist activity and others.

Immunoreactivities for VIP (Jancsó et al., 1981; see Holzer, 1991), PACAP (Moller et al., 1993; see Sundler et al., 1996) and NO synthase (or NADPH-diaphorase activity) seem to be present in primary afferent neurons (Aimi et al., 1991; Bscheidl et al., 1994; Papka et al., 1995; Zheng et al., 1997). Unexpectedly, however, these immunoreactivities can be increased by peripheral nerve injury (McGregor et al., 1984; see Sundler et al., 1996 for review) and also by the capsaicin-like substance resiniferatoxin (Farkas-Szállási et al., 1995; Vizzard et al., 1995). On the other hand, capsaicin pretreatment may decrease neuronal nitric oxide synthase (Ren and Ruda, 1995; Zheng et al., 1999), VIP (see Holzer, 1991) or PACAP (Moller et al., 1993) immunoreactivities in primary afferents. Moreover, capsaicin pretreatment can counteract the axotomy-induced increase in nitric oxide synthase expression (Ren and Ruda, 1995). Immunochemical (Maggi et al., 1989; Wang et al., 1995; Zhang et al., 1997) and functional data (Merhi et al., 1998; Szikszay et al., 1998; Towler et al., 1998) also support the idea that VIP, PACAP or NO may be released from primary afferents and contribute to the effects of these neurons at the periphery and, possibly, in the central nervous system. PACAP might also function as a positive modulator of neuropeptide release in sensory nerves (Wang et al., 1995).

The lack of effect of the VIP/PACAP receptor antagonist PACAP-(6–38) or the NO synthase blocker L-NOARG on the excitatory effect of capsaicin in the ileum, however, does not support the idea that VIP or NO might be involved in the capsaicin-evoked response. With the possible involvement of PACAP, this type of evidence is weaker because PACAP-(6–38) only moderately reduced the contractile action of exogenous PACAP (while it abolished that of VIP). The strong inhibition of the relaxant effect of PACAP in the ileum by PACAP-(6–38) confirms the PACAP receptor antagonist action of this fragment in the guinea-pig small intestine. This also implies that neuronal and smooth muscle receptors for PACAP in the guinea-pig ileum are, at least in part, different.

In conclusion, an ATP-like endogenous P_2 purinoceptor ligand, possibly of sensory neuronal origin is involved in the excitation of myenteric cholinergic neurons in the course of the motor response to capsaicin in the guinea-pig small intestine. On the other hand, no evidence has been found for an involvement of NO or a VIP/PACAP-like peptide in this response.

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