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# Effect of vanilloid drugs on gastrointestinal transit in mice

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**1** We have studied the effect of capsaicin, piperine and anandamide, drugs which activate vanilloid receptors and capsazepine, a vanilloid receptor antagonist, on upper gastrointestinal motility in mice.

**2** Piperine (0.5–20 mg kg$^{-1}$ i.p.) and anandamide (0.5–20 mg kg$^{-1}$ i.p.), dose-dependently delayed gastrointestinal motility, while capsaicin (up to 3 mg kg$^{-1}$ i.p.) was without effect. Capsazepine (15 mg kg$^{-1}$ i.p.) neither *per se* affected gastrointestinal motility nor did it counteract the inhibitory effect of both piperine (10 mg kg$^{-1}$) and anandamide (10 mg kg$^{-1}$).

**3** A *per se* non effective dose of SR141716A (0.3 mg kg$^{-1}$ i.p.), a cannabinoid $CB_1$ receptor antagonist, counteracted the inhibitory effect of anandamide (10 mg kg$^{-1}$) but not of piperine (10 mg kg$^{-1}$). By contrast, the inhibitory effect of piperine (10 mg kg$^{-1}$) but not of anandamide (10 mg kg$^{-1}$) was strongly attenuated in capsaicin (75 mg kg$^{-1}$ in total, s.c.)-treated mice.

**4** Pretreatment of mice with $N^G$-nitro-L-arginine methyl ester (25 mg kg$^{-1}$ i.p.), yohimbine (1 mg kg$^{-1}$, i.p.), naloxone (2 mg kg$^{-1}$ i.p.), or hexamethonium (1 mg kg$^{-1}$ i.p.) did not modify the inhibitory effect of both piperine (10 mg kg$^{-1}$) and anandamide (10 mg kg$^{-1}$).

**5** The present study indicates that the vanilloid ligands anandamide and piperine, but not capsaicin, can reduce upper gastrointestinal motility. The effect of piperine involves capsaicin-sensitive neurones, but not vanilloid receptors, while the effect of anandamide involves cannabinoid $CB_1$, but not vanilloid receptors.

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**Keywords:** Anandamide; piperine; capsazepine; primary afferent neurones; vanilloid receptors; cannabinoid receptors; capsaicin; intestinal motility

**Abbreviations:** DMSO, dimethyl sulphoxide; L-NAME, $N^G$-nitro-L-arginine methyl ester; VR1, vanilloid receptor (subtype 1)

## Introduction

A subpopulation of primary afferent neurones has been characterized by using the sensory neurotoxin capsaicin (Maggi & Meli, 1988), the active ingredient of chilli (from the *Capsicum* family). These neurones are small, 'dark' and type 'B', and give rise to unmyelinated afferent fibres (Torsoli *et al*., 1993). Capsaicin-sensitive sensory neurones can modulate intestinal motility as they convey signals coming from the gastrointestinal tract to the central nervous system and may simultaneously release transmitters (from the same terminal which is activated by an adequate stimulus) able to affect enteric neurotransmission (Holzer, 1991).

The action of capsaicin on afferent neurones is traditionally regarded as involving two phases: an acute excitatory effect which lead to transmitter release, followed by desensitization and damage after prolonged or repeated exposure (Holzer, 1991). In recent years it has been shown that the action of capsaicin on afferent neurones can be mediated through activation of specific receptors, namely vanilloid receptors (Caterina *et al*., 1997; Tominaga *et al*., 1998). Vanilloid receptors can be also activated by other irritant principles present in 'hot' spices, such as piperine, the active ingredient of black pepper (*Piper nigrum*) and zingerone, isolated from ginger (*Zingiber officinalis*) (Liu & Simon, 1997; Sterner & Szallasi, 1999). Capsaicin, piperine and gingerone are structurally similar, as they share a vanillyl moiety essential for bioactivity (Sterner & Szallasi, 1999). A functional vanilloid receptor (VR1) has been cloned (Caterina *et al*., 1997) and a vanilloid receptor antagonist, namely capsazepine, is available for pharmacological characterization (Sterner & Szallasi, 1999). VR1 is a cation channel that is expressed in a major sub-group of small 'dark' neurones of the dorsal root, trigeminal and vagal sensory ganglia (Caterina *et al*., 1997; Helliwell *et al*., 1998; Sterner & Szallasi, 1999; Szolcsanyi, 2000) and in several brain areas (Sasamura *et al*., 1998). The discovery of vanilloid receptors suggests the existence of endogenous vanilloid receptor ligands; in fact, the first of such vanilloids has identified as anandamide (arachidonylethanolamide) (Zygmunt *et al*., 1999; Smart *et al*., 2000), originally isolated as an endogenous cannabinoid receptor ligand (Devane *et al*., 1992). These findings suggest the existence of endogenous vanilloid receptor modulators lacking a vanillyl motif (Sterner & Szallasi, 1999).

Given the importance of primary afferent neurones in the control of intestinal motility *in vivo* and since vanilloid receptors are highly expressed on these neurones (Szallasi & Blumberg, 1999), we have evaluated the effect vanilloid drugs on upper gastrointestinal transit in mice. We have used anandamide, capsaicin and piperine, which activate vanilloid

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receptors (Liu & Simon, 1997; Sterner & Szallasi, 1999; Zygmunt *et al*., 1999) and capsazepine, a vanilloid receptor antagonist (Bevan *et al*., 1992).

## Methods

### *Animals*

Male ICR mice (Harlan Italy, Corezzana, MI, U.S.A.) (20–22 g) were used after 1 week of acclimation (temperature 23±2°C; humidity 60%). Food was withheld 3 h before experiments but there was free access to drinking water.

### *Upper gastrointestinal transit*

Gastrointestinal transit was measured as previously described (Izzo *et al*., 1999; 2000b). Mice received orally a black marker (10% charcoal suspension in 5% gum arabic, 0.1 ml 10 g mouse$^{-1}$) and 20 min later the mice were killed by asphyxiation with $CO_2$ and the gastrointestinal tract removed. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum.

Capsaicin (0.1–3 mg kg$^{-1}$), piperine (0.5–20 mg kg$^{-1}$), anandamide (0.5–20 mg kg$^{-1}$), were given (i.p.) 20 min before charcoal administration. In some experiments capsaicin (3 mg kg$^{-1}$ i.p.) was given immediately ($t=0$) or 10 min ($t=10$) before charcoal administration.

In some experiments capsazepine (15 mg kg$^{-1}$), SR141716A (0.3 mg kg$^{-1}$), yohimbine (1 mg kg$^{-1}$), naloxone (2 mg kg$^{-1}$), hexamethonium (1 mg kg$^{-1}$) or N$^{G}$-nitro-L-arginine methyl ester (L-NAME 25 mg kg$^{-1}$) were given (i.p.) 30 min before piperine (10 mg kg$^{-1}$) or anandamide (10 mg kg$^{-1}$). The dose of capsazepine was selected on the basis of preliminary experiments: capsazepine (15 mg kg$^{-1}$ i.p.) prevented the antinociceptive effect of capsaicin (3 mg kg$^{-1}$ i.p.) in the hot plate test model (Perkins & Campbell, 1992). The other doses were selected on the basis of previous published work (Santos & Rao, 1999; Izzo *et al*., 1994).

In another set of experiments, the effect of anandamide (10 mg kg$^{-1}$ i.p.) and piperine (10 mg kg$^{-1}$ i.p.) was evaluated in capsaicin-treated animals: mice were anaesthetized with sodium pentobarbital (30 mg kg$^{-1}$) and treated with increasing doses of capsaicin for 2 consecutive days (25 and 50 mg kg$^{-1}$) to deplete neuropeptides in primary afferent neurones (Matsuda *et al*., 1999). To counteract any respiratory impairment associated with administration of capsaicin, the mice were pretreated with aminophylline (10 mg kg$^{-1}$) 30 min before capsaicin injection. After 14 days, the efficacy of capsaicin treatment was assessed by the eye-wiping test (Holzer *et al*., 1990): impaired chemosensitivity of corneal afferents which are no longer sensitive to a solution of 1% $NH_4OH$.

### *Drugs*

Drugs used were: anandamide (soya oil/water emulsion), capsaicin, capsazepine (Tocris Cookson, Bristol, U.K.), aminophylline, hexamethonium bromide, piperine, naloxone hydrochloride, N$^{G}$-nitro-methyl arginine methyl ester (L-NAME) hydrochloride, yohimbine hydrochloride, (SIGMA, Milan, Italy), sodium pentobarbital (Carlo Sessa), SR141716A [(N-piperidin-1-yl)-5-(4-chlorophenyl)-1-2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride was a gift from Dr Madaleine Mossè (SANOFI-Recherche, Montpellier, France). Capsaicin, capsazepine and piperine were dissolved in dimethyl sulphoxide (DMSO), while the other drugs were dissolved in saline.

### *Statistics*

Data are means±s.e.mean. To determine statistical significance, Student's *t*-test for unpaired data or one-way analysis of variance followed by Tukey-Kramer multiple comparisons test was used. A $P$ value less than 0.05 was considered significant.

## Results

Administration of piperine (0.5–20 mg kg$^{-1}$) or anandamide (0.5–20 mg kg$^{-1}$) produced a dose-dependent delay in upper gastrointestinal transit (Figure 1). This effect was significant (from both compounds) starting from the dose of 10 mg kg$^{-1}$. By contrast capsaicin (up to 3 mg kg$^{-1}$ i.p.) did not modify significantly intestinal motility (transit: control 48±5%, capsaicin 0.1 mg kg$^{-1}$ 45±5%, capsaicin 0.3 mg kg$^{-1}$ 40±3%, capsaicin 1 mg kg$^{-1}$ 39±8, capsaicin 3 mg kg$^{-1}$ 45±4%, $n=7-8$ for each experimental group, $P>0.2$). When capsaicin (3 mg kg$^{-1}$ i.p.) was given immediately ($t=0$) or 10 min ($t=10$) before charcoal administration, it did not modify significantly intestinal motility (transit at $t=0$: control 46±4%; capsaicin 41±3%; transit at $t=10$: control 49±5, capsaicin 46±4, $n=7-8$ for each experimental group, $P>0.2$)

Upper gastrointestinal transit was not significantly modified by pretreatment (15 mg kg$^{-1}$ i.p.) with the vanilloid

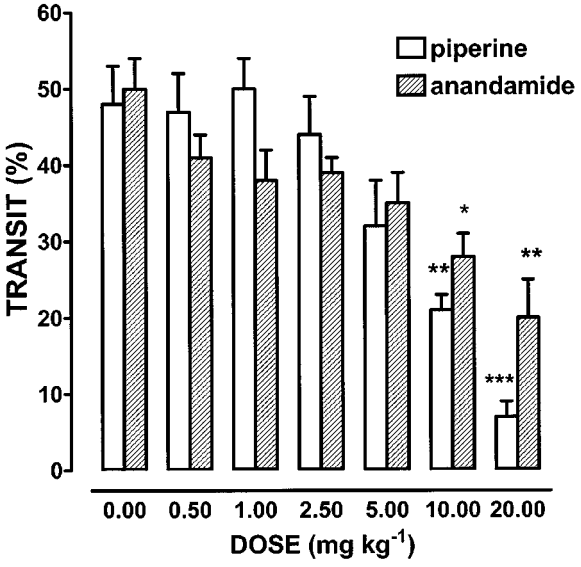


**Figure 1** Effect of i.p.-injected anandamide and piperine on upper gastrointestinal transit. Each point represents the mean±s.e.mean of six animals for each experimental group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs corresponding control.

receptor antagonist capsazepine (transit: control 52±4%, capsazepine 42±5%, $n=8$ for each experimental group, $P>0.2$). Lower doses of capsazepine (1, 3 and 10 mg kg$^{-1}$) were also ineffective (data not shown).

The cannabinoid $CB_1$ receptor antagonist SR141716A (0.3 mg kg$^{-1}$), but not the vanilloid receptor antagonist capsazepine (15 mg kg$^{-1}$) counteracted the inhibitory effect of anandamide (10 mg kg$^{-1}$) (Figure 2). However, both capsazepine or SR141716A did not modify the inhibitory effect of piperine (10 mg kg$^{-1}$) (Figure 2). Vehicle (DMSO 5 $\mu$l) for SR141716A or capsazepine did not modify significantly anandamide (10 mg kg$^{-1}$ i.p.)- or piperine (10 mg kg$^{-1}$ i.p.)-induced changes in motility (per cent transit: control 50±4, anandamide 27±6, anandamide+DMSO 25±5, piperine 23±3, piperine+DMSO 21±5, $n=7-8$ for each experimental group).

As shown in Figure 3, pretreatment of mice with capsaicin (75 mg kg$^{-1}$ in total, 13 and 14 days before) did not modify significantly gastrointestinal transit. However, piperine (10 mg kg$^{-1}$), but not anandamide (10 mg kg$^{-1}$) was without significant effect in mice pretreatment with capsaicin (Figure 3). Vehicle (DMSO 5 $\mu$l) for capsaicin did not modify significantly anandamide (10 mg kg$^{-1}$ i.p.)- or piperine (10 mg kg$^{-1}$ i.p.)-induced changes in motility (per cent transit: control 49±5, anandamide 28±5, anandamide+DMSO 24±5, piperine 24±4, piperine+DMSO 22±6, $n=7-8$ for each experimental group)

The inhibitory effect of both piperine (10 mg kg$^{-1}$) and anandamide (10 mg kg$^{-1}$) was unchanged in mice pretreated with naloxone (2 mg kg$^{-1}$), yohimbine (1 mg kg$^{-1}$), L-NAME (25 mg kg$^{-1}$) or hexamethonium (1 mg kg$^{-1}$) (Figure 4).

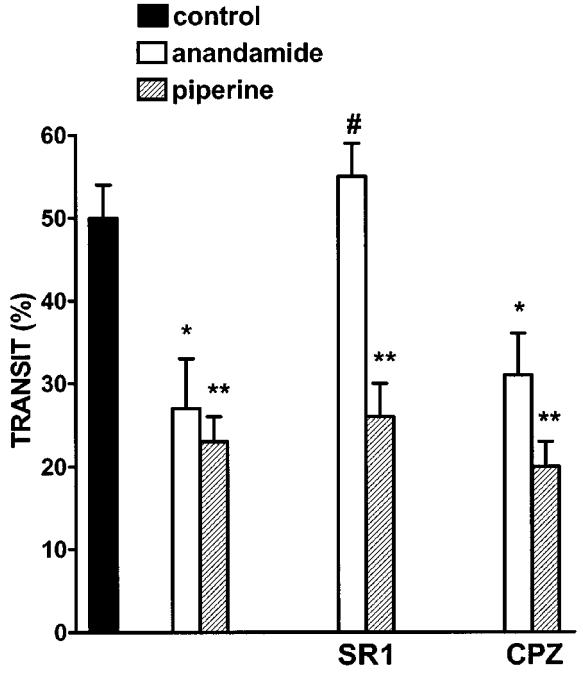


**Figure 2** Effect of piperine (10 mg kg$^{-1}$, i.p.) or anandamide (10 mg kg$^{-1}$) on upper gastrointestinal transit alone or in mice treated with the cannabinoid $CB_1$ receptor antagonist SR141716A (SR1, 0.3 mg kg$^{-1}$ i.p.) or the vanilloid receptor antagonist capsazepine (CPZ, 15 mg kg$^{-1}$, i.p.). Results are mean±s.e.mean of 7–8 animals for each experimental group. $*P<0.05$ and $**P<0.01$ vs control and $\#P<0.01$ vs anandamide (alone).

At the dosage used, none of the antagonists tested, i.e. SR141716A, yohimbine, naloxone, hexamethonium, L-NAME, had any significant effect *per se* on upper

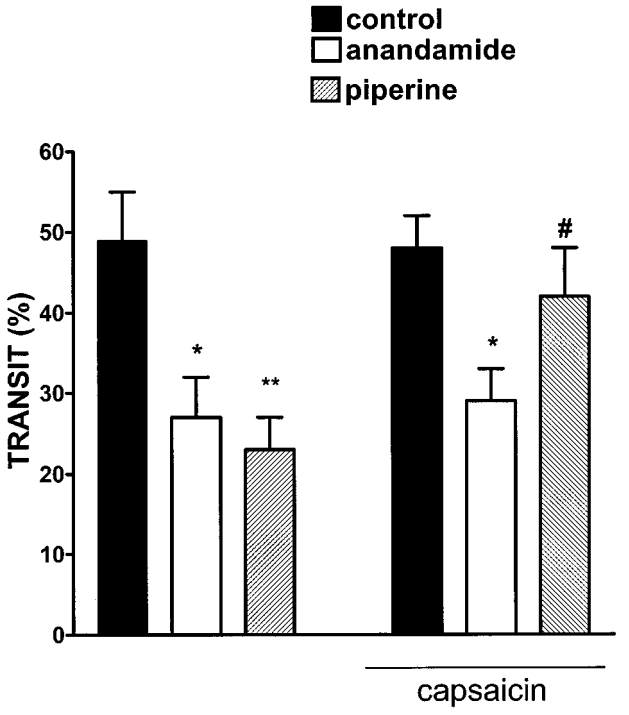


**Figure 3** Effect of piperine (10 mg kg$^{-1}$ i.p.) or anandamide (10 mg kg$^{-1}$ i.p.) on upper gastrointestinal transit in mice not receiving capsaicin or in capsaicin (75 mg kg$^{-1}$ s.c., 13 and 14 days before)-treated mice. Each point represents the mean±s.e.mean of 7–8 animals for each experimental group. $*P<0.05$ and $**P<0.01$ vs corresponding control (animals not treated with anandamide or piperine) and $\#P<0.05$ vs piperine.

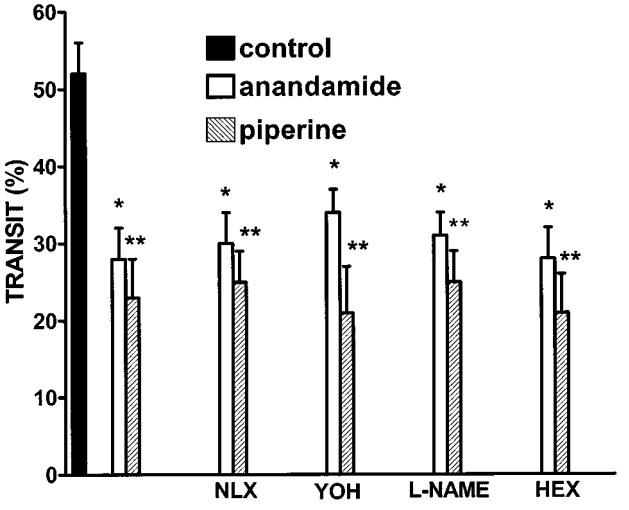


**Figure 4** Effect of piperine (10 mg kg$^{-1}$, i.p.) or anandamide (10 mg kg$^{-1}$ i.p.) on upper gastrointestinal transit alone or in mice treated with naloxone (NLX, 2 mg kg$^{-1}$, i.p.), yohimbine (YOH, 1 mg kg$^{-1}$ i.p.), $N^G$-nitro-L-arginine methyl ester (L-NAME, 25 mg kg$^{-1}$) and hexamethonium (HEX, 1 mg kg$^{-1}$ i.p.). Results are mean±s.e.mean of 7–8 animals for each experimental group. $*P<0.05$ and $**P<0.01$ vs control.

gastrointestinal transit (variation of upper gastrointestinal transit: SR141716A $+16\pm6\%$, yohimbine $+14\pm8$, naloxone $+2\pm5\%$, hexamethonium $+15\pm7\%$, L-NAME $-8\pm6\%$, $n=7-8$, $P>0.2$).

DMSO (10 $\mu$l mouse$^{-1}$) *per se* did not significantly modify gastrointestinal transit (per cent transit control $51\pm5$, DMSO $48\pm4$, $n=6$, $P>0.2$).

## Discussion

Primary afferent neurones carry sensory information to the central nervous system and may simultaneously release neurotransmitters able to affect enteric neurotransmission (Torsoli *et al*., 1993; Goyal & Hirano, 1996). In recent years it has been shown that capsaicin and other active ingredient present in 'hot spices' bind to specific receptors, named vanilloid receptors, mostly located on the cell membrane of primary afferent neurones (Szallasi & Blumberg, 1999). Previous investigators have shown that piperine and capsaicin, which act *via* vanilloid receptors (Sterner & Szallasi, 1999), affect gastric and intestinal motility *in vitro* (Maggi *et al*., 1986a,b; Jin *et al*., 1990; Takaki *et al*., 1990; Lefebvre *et al*., 1991; Allesher *et al*., 1992; Holzer-Petsche *et al*. 1989; Barthò *et al*., 2000). In addition, the endocannabinoid anandamide, which is know to inhibit enteric excitatory transmission *in vitro* (Izzo *et al*., 1998) and intestinal motility *in vivo* (Fride, 1995; Calignano *et al*., 1997), has been recently identified as an endogenous vanilloid ligand (Zygmunt *et al*., 1999; Smart *et al*., 2000). These results could suggest an involvement of vanilloid receptors in the control of intestinal motility.

In the present study, we have shown that piperine and anandamide, which activate vanilloid receptors (Liu & Simon, 1997; Zygmunt *et al*., 1999; Smart *et al*., 2000), are able to produce a dose-dependent reduction of upper gastrointestinal transit. However, it is unlikely that this effect is due to activation of vanilloid receptors as the inhibitory effect of both piperine and anandamine was not modified by capsazepine, a specific vanilloid receptor antagonist. In addition, capsaicin (up to 3 mg kg$^{-1}$), another vanilloid receptor agonist (Sterner & Szallasi, 1999) did not affect upper gastrointestinal transit thus confirming the lack of involvement of vanilloid receptors in the control of upper gastrointestinal transit. Higher doses of capsaicin (i.p.) were not studied as they were toxic. Others have shown that lower doses of capsaicin ($<3$ mg kg$^{-1}$) affected gastric motility (Kang *et al*., 1993) and gastric blow flow (Abdel Salam *et al*., 1996) in rats.

Previous investigators have shown that chronic treatment with capsaicin (to ablate capsaicin-sensitive afferent neurons) does not affect gastrointestinal propulsion in physiological states, while it reduced the inhibition of gastrointestinal transit due to surgical trauma or peritoneal administration (Holzer, 1986; Holzer *et al*., 1987). When given acutely (as in our study) capsaicin reduced intestinal transit in the rat (Miller *et al*., 1981; Chang *et al*., 1999). In the present study performed in the mouse, capsaicin did modify upper gastrointestinal transit. The use of a different animal specie (rat *vs* mouse) or different regions of the gut studied (intestinal transit *vs* gastrointestinal transit) could explain the discrepancy between our results (no effect of capsaicin on motility) and those reported in the rat (delaying effect of capsaicin on motility). However, others have shown that capsaicin did not modify upper gastrointestinal transit in the rat (Kang *et al*. 1993).

Capsaicin has been used systematically to ablate all capsaicin-sensitive C fibres to produce sensory pathway-specific ablation in various animal species, including the mouse (Barrachina *et al*., 1997). In the digestive tract, capsaicin-sensitive afferent innervation participates in nociception, gastroprotection and intestino-intestinal activation of inhibitory reflexes (Holzer, 1991; Holzer *et al*., 1991). Gastroprotection by oral capsaicin could result from increase of mucosal blood flow and inhibition of gastric motility (Takeuchi *et al*., 1991). In order to verify whether the vanilloid drugs-induced changes in motility were due to an effect on capsaicin-sensitive nerve terminals, the effect of anandamide and piperine was evaluated in mice desensitized by systemic capsaicin doses. Thus, we have shown that the inhibitory effect of piperine, but not anandamide, was markedly attenuated by the pretreatment with capsaicin (13 and 14 days before). These results suggests that capsaicin-sensitive sensory nerves are, at least in part, involved in the inhibition of intestinal transit by piperine. Consistent with these *in vivo* results, Takaki *et al*. (1990) have shown that pretreatment of the isolated ileum with capsaicin prevented piperine-induced motility changes. If we assume that piperine modify gastrointestinal motility by acting on capsaicin-sensitive neurones, at present it is not clear why capsaicin, which, like piperine, acts on capsaicin-sensitive neurones does not affect intestinal motility; thus, we can hypothesize that there are sites on sensory neurones which are selectively recognized by piperine, but not by capsaicin. Activation of these sites can delay gastrointestinal motility and their identification could represent a novel target for therapeutic drugs. It is unlikely that the vanillyl moiety is essential for this activity as both capsaicin and piperine share this chemical group. Therefore, the different pharmacological response evoked by capsaicin and piperine in this study could be explained, at least in part, by the existence of different subtypes of vanilloid receptors (Acs *et al*., 1997; Szolcsanyi, 2000) or perhaps by the fact that both capsaicin and piperine possess non-specific actions (i.e. not restricted to primary afferent neurones) on nerves and smooth muscle (Holzer, 1991; Takaki *et al*., 1990). It is unlikely that the difference between capsaicin and piperine is due to a different activation and desensitisation kinetics (i.e. effect of capsaicin, due to a more rapid desensitization, is much shorter-lasting than that of piperine) as capsaicin was inactive even when given immediately ($t=0$) or 10 min ($t=10$) before charcoal administration.

Activation of prejunctional cannabinoid $CB_1$ is known to inhibit enteric excitatory transmission (Pertwee *et al*., 1996; Izzo *et al*., 1998) and peristalsis (Heinemann *et al*., 1999; Izzo *et al*., 2000a) in the isolated guinea-pig ileum. Cannabinoid receptor agonists reduce while the cannabinoid receptor antagonist SR141716A increase intestinal motility *in vivo* by activating peripheral (enteric) cannabinoid $CB_1$ receptors (Izzo *et al*., 2000b). In the present study we have shown that the effect of anandamide, but not of piperine, was counteracted by a *per se* non effective dose of the selective cannabinoid $CB_1$ antagonist SR141716A (Rinaldi-Carmona *et al*., 1995), indicating an involvement of cannabinoid $CB_1$

receptors in the inhibitory effect of anandamide. However, other prejunctional or presynaptic systems, such as opioid or $\alpha_2$-adrenergic receptors, which are known to be involved in the regulation of intestinal motility (Dockray, 1994; Burks, 1994) are not involved in the inhibitory effect of the two vanilloid ligands as the inhibitory effect of both anandamide and piperine was not modified by naloxone, an opioid receptor antagonist or yohimbine, an $\alpha_2$-adrenergic receptor antagonist.

Previous investigators have shown that capsaicin can depress intestinal peristalsis with a mechanism involving nitric oxide (Bartho & Holzer, 1995); in addition, anandamide stimulates nitric oxide production in neural tissues (Stefano *et al*., 1997). Nitric oxide is now recognized as perhaps the major mediator of relaxation induced by enteric inhibitory neurones (Burks, 1994). Reduction of gastrointestinal motility *in vivo* can either results from inhibition of nitric oxide synthesis or from formation of excess nitric oxide (Orihata & Sarna, 1994; De Winter *et al*., 1997). In the present study we have shown that L-NAME, a nitric oxide synthase inhibitor, at dose previously shown to be effective (Izzo *et al*., 1994), was not able to modify the inhibitory effect of both anandamide and piperine. Thus, an involvement of nitric oxide in the motility changes associated with these vanilloid drugs seems unlikely.

In conclusion, the present study indicates that vanilloid ligands have a different effect on intestinal motility. Piperine may decrease gastrointestinal transit through actions on capsaicin-sensitive neurones; anandamide decreases intestinal motility through an action on cannabinoid $CB_1$ receptors (but not on vanilloid receptors or on capsaicin-sensitive neurones) while capsaicin, another activator of vanilloid receptors, was without effect. The effect of both anandamide and piperine probably involves peripheral mechanisms (as suggested by the lack of effect of hexamethonium) but does not involve nitric oxide generation or activation of $\alpha_2$-adrenergic or opioid receptors. The lack of effect of the vanilloid receptor antagonist capsazepine to affect *per se* intestinal motility or to counteract the inhibitory effect of the vanilloid ligands (together with the ineffectiveness of the vanilloid receptor agonist capsaicin) suggests that endogenous or exogenous activation of vanilloid receptors does not play a role in the control of upper gastrointestinal transit under physiological states.

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