Tachykininergic transmission to the circular muscle of the guinea-pig ileum: evidence for the involvement of NK₂ receptors

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1 The effect of newly developed, receptor-selective tachykinin antagonists (GR 71,251 for NK₁ receptors, MEN 10,376 and L 659,877 for NK₂ receptors) on noncholinergic transmission to the circular muscle of the guinea-pig ileum has been investigated.

2 In circular muscle strips of the ileum, electrical field stimulation in the presence of atropine (2 µM) and apamin (0.1 µM) evoked a complex motor response. The tonic primary contraction in this response was reduced by GR 71,251 (10 µM) and MEN 10,376 (3–10 µM) but not by L 659,877 (up to 10 µM). The presence of apamin was necessary in this experimental arrangement to unmask an atropine-resistant primary contraction, sensitive to tachykinin antagonists. The motor response was abolished by tetrodotoxin.

3 In circular strips of the ileum GR 71,251 (10 µM) inhibited the tonic contraction produced by [Sar⁹] substance P sulphone, a selective NK₁ receptor agonist but not that produced by [β Ala⁸] neurokinin A (4–10), a selective NK₂ receptor agonist. By contrast, MEN 10,376 antagonized the effect of the NK₂ agonist while leaving the response to the NK₁ agonist unaffected.

4 In whole segments of the ileum, distension of the gut wall by an intraluminal balloon placed at about 1 cm from the point of recording of mechanical activity of the circular muscle produced atropine-sensitive phasic contractions (ascending enteric reflex). In the presence of atropine (2 µM), a noncholinergic response was elicited, which required larger volumes of distension than the cholinergic one. The atropine-resistant ascending enteric reflex was enhanced by apamin (0.1 µM) and abolished by tetrodotoxin, either in the presence or absence of apamin.

5 MEN 10,376 (3–10 µM) inhibited the atropine-resistant ascending enteric reflex in the presence of apamin while GR 71,251 or L 659,877 (10 µM each) were ineffective. MEN 10,376 inhibited the atropine-resistant ascending enteric reflex to a larger extent in the absence than in the presence of apamin and also slightly inhibited the ascending enteric reflex in the absence of atropine.

6 These findings provide evidence for an involvement of NK₂ tachykinin receptors in excitatory transmission to the circular muscle of the guinea-pig ileum. NK₂ receptors are also involved in the physiological-like circular muscle activation produced by stimulation of intramural neuronal pathways which subserve the atropine-resistant ascending enteric reflex.

Keywords: Tachykinins; tachykinin receptors; guinea-pig ileum; circular muscle; ascending enteric reflex

Introduction

Ample evidence, based on pharmacological, physiological, anatomical and neurochemical findings, indicates that tachykinins (TKs) play a physiological role as excitatory transmitters in the guinea-pig ileum (Franco *et al.*, 1979; Costa *et al.*, 1981; 1985a,b; Bartho *et al.*, 1982a,b; Donnerer *et al.*, 1984; Smith & Furness, 1988; Llewellyn-Smith *et al.*, 1988; 1989; Bartho *et al.*, 1989; Holzer, 1989; see also Bartho & Holzer, 1985 for review). In the guinea-pig small intestine, substance P and other TKs, such as neurokinin A (NKA) are present (Too *et al.*, 1989).

The longitudinal contractile response of the guinea-pig ileum to TKs is mediated by specific receptors (Lee *et al.*, 1982). There is evidence that NK₁ receptors mediate the direct response of muscle cells while NK₃ receptors activate intramural effector neurones which in turn release acetylcholine and possibly endogenous TKs (Kilbinger *et al.*, 1986; Laufer *et al.*, 1986; 1988; Guard & Watson, 1987). In spite of its importance for the genesis of propulsive activity and peristalsis, the effects of TKs on the circular muscle have been studied less (Holzer *et al.*, 1980; Costa *et al.*, 1985a,b). By use of natural TKs and receptor-selective TK agonists, evidence has been presented that NK₁, NK₂ and NK₃ receptors mediate the spasmogenic action of TKs in the circular

muscle of the guinea-pig ileum (Maggi *et al.*, 1990a); the response to NK₂ receptor stimulation seems to be entirely ascribable to direct smooth muscle activation, that produced by NK₃ receptor stimulation is entirely neurogenic while that produced by NK₁ receptor stimulation has intermediate characteristics.

Pharmacological evidence for an involvement of *endogenous* TKs in atropine-resistant peristalsis of the ileum comes from studies using TK antagonists of the first generation, such as Spantide I or [D-Pro², D-Trp^{7,9}] substance P. These substances have been shown to inhibit atropine-resistant, nerve-mediated longitudinal contractions (Björkroth, 1983; Bartho *et al.*, 1983) as well as the atropine-resistant components of the peristaltic reflex and of the ascending enteric reflex (Bartho *et al.*, 1982a,b; Costa *et al.*, 1985a,b; Grider, 1989; Holzer, 1989).

However, TK antagonists of the first generation have little if any ability to discriminate between different TK receptors (Regoli, 1985; Buck & Shatzner, 1988) and consequently, the relative role of substance P (SP) and NKA in the overall response cannot be deduced. With the introduction of new, second generation TK antagonists, specific for different types of TK receptors, it has become possible to obtain more information about the relative contributions of different receptor types and, consequently, of the various TKs, in nerve-mediated responses in different tissues (Maggi *et al.*, 1991a,b). The aim of this study was to address the question

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of the relative role of NK₁ and NK₂ receptors in the physiological activation of the circular muscle of the guinea-pig ileum by using some of the newly developed receptor-selective antagonists to block atropine-resistant motor responses produced by nerve activation.

Methods

General

Male albino guinea-pigs (250–300 g) were stunned and bled. A segment of the ileum was excised and placed in oxygenated (95% O₂ and 4% CO₂) Krebs solution.

Circular muscle strips

The ileum was opened along the mesenteric border and pinned flat on a Petri dish. A small strip (approximately 3 mm wide) was then dissected along the circular axis as described by Costa *et al.* (1985b) with the only exception that no attempt was made to remove the mucosal layer, in order to avoid any possible damage to the inner circular muscle. The strips were transferred to 5 ml baths for isotonic recording (load 3 mN) of mechanical activity via a Basile 7050 Unirecord. In some experiments, the strips were electrically stimulated by means of two platinum wire electrodes (distance between electrodes, 5 cm) placed at the top and bottom of the organ bath, connected to a GRASS S88 stimulator. All experiments started after a 120 min equilibration period. In a first series of experiments, three different receptor-selective tachykinin antagonists, GR 71251 (NK₁ receptor selective, Hagan *et al.*, 1990; Hall & Morton, 1991), MEN 10,376 (NK₂ receptor selective, Maggi *et al.*, 1991c) and L 659,877 (NK₂ receptor selective, Williams *et al.*, 1988) were studied on the noncholinergic (atropine-resistant) contraction produced by electrical stimulation (5 Hz for 30 s, 0.15 ms pulse width, maximal voltage) in the presence of atropine (2 µM) and apamin (0.1 µM). Apamin was added to the bath in order to unmask a primary atropine-resistant contraction to field stimulation (see Results).

After having recorded two or more reproducible responses to electrical stimulation at 20 min intervals, the stated concentration of the antagonist was added to the bath and left in contact for 15 min.

In a second series of experiments, the selectivity of antagonists was tested against circular muscle contractions produced by the NK₁ receptor-selective agonist [Sar⁹] substance P sulphone (Drapeau *et al.*, 1987) or the NK₂ receptor-selective agonist, [βAla⁸] neurokinin A (4–10) (Rovero *et al.*, 1989; Maggi *et al.*, 1990b). The concentration of the two agonists (300 nM) was selected in order to produce a contraction comparable to that produced by electrical stimulation. Experiments were performed in the presence of atropine (2 µM) and apamin (0.1 µM).

In a third series of experiments the effects of GR 71,251 and MEN 10,376 toward field stimulation-induced contraction were investigated in the absence of atropine and apamin. Stimulation of 10 Hz for 30 s resulted in a primary contraction comparable to that observed in the presence of atropine and apamin. All contractile responses were expressed as % of maximal response to 160 mM KCl.

Ascending enteric reflex

The effect of TK antagonists on the ascending enteric reflex produced by radial stretch (balloon distension) was studied in 6–7 cm long segments of whole ileum by use of an experimental arrangement similar to that described by Holzer (1989). The ileal segment was secured horizontally at the bottom of a 6 ml flat glass organ bath by a metal bar passed through the lumen. The bath was filled with oxygenated Krebs solution and warmed to 37°C. The mechanical activity

of the circular muscle was recorded via a thread secured to the mesentery close to the intestinal wall. The thread connected the ileum to an auxotonic transducer and the preparation was kept under a resting load of 5 mN. The ascending enteric reflex was elicited either in the absence or in the presence of atropine (2 µM) and, in atropine-treated preparations either in the presence or absence of apamin (0.1 µM). Distension was carried out by inflating a latex balloon (Hugo Sachs, size 5, unloaded volume 100 µl) placed at about 1 cm caudal to the recording site; for experiments without atropine a volume of 0.1 ml was sufficient to elicit maximal responses, while in the presence of atropine larger volumes (0.2–0.3 ml) were required. After having recorded at least 3 reproducible responses to balloon distension (10–15 min apart from each other) drugs were added to the bath. The neurogenic nature of the evoked response was checked at the end of experiments by addition of tetrodotoxin (1 µM). Amplitude of evoked responses was expressed as % of the response to 80 mM KCl which produced maximal circular shortening of the preparation. All the responses evoked by balloon distension, either in the presence or absence of atropine or apamin were abolished by tetrodotoxin.

Statistical analysis

All data in the text are mean ± standard error (s.e.) of the mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by analysis of variance, when applicable.

Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany), tetrodotoxin (Sankyo, Tokyo, Japan), apamin (Sigma). The NK₂-selective antagonist L 659,877 (cyclo[Leu-Met-Gln-Trp-Phe-Gly]) was purchased from Cambridge Research Biochemicals, England. [βAla⁸]-NKA (4–10) and MEN 10,376 were synthesized by Dr P. Rovero, Chemistry Department, Menarini Pharmaceuticals, by conventional solid-phase methods. GR 71,251 ([D-Pro⁹][Spiro-γ-lactam]Leu¹⁰,Trp¹¹]SP (1–11)) was kindly provided by Dr R. Hagan, Department of Neuropharmacology, Glaxo Group Research, Ware, England.

Concentrated solutions (1–10 mM) of L 659,877, MEN 10,376 and [βAla⁸]-neurokinin A(4–10) were prepared in dimethylsulphoxide (DMSO) and diluted in Krebs solution. Control experiments showed that DMSO alone (0.1–0.3% final concentration) had no effect on the responses under study. The other peptides were dissolved in Krebs solution. Concentrations of L 659,877 greater than 10 µM were not tested because of precipitation in the bath.

Results

Circular muscle strips: response to electrical stimulation

In the presence of atropine (2 µM), electrical field stimulation (1–20 Hz) usually failed to evoke a primary contraction of circular muscle strips (Figures 1 and 2). During stimulus delivery either a slight relaxation was observed in some strips or no effect at all. A large 'off' contraction was observed at the end of the stimulus period (30 s). Preliminary experiments showed that the 'off' contraction was hardly affected by tachykinin antagonists, although it was suppressed by tetrodotoxin. Since apamin has been reported to exert a facilitatory action on the putatively tachykininergic excitation of circular muscle (Holzer *et al.*, 1989), its effect was investigated. Apamin (0.1 µM) induced or increased submaximal phasic contractions in nearly all preparations tested; this effect was long-lasting.

In the presence of apamin, an atropine-resistant primary contraction to field stimulation was unmasked. A typical response to 1–10 Hz field stimulation (see Figures 1 and 2)

Figure 1 Typical tracing showing the response of circular muscle strips from the guinea-pig ileum to electrical field stimulation (5 Hz for 30 s, horizontal bars) in the absence and presence of atropine and unmasking of an atropine-resistant primary contraction by apamin. Tetrodotoxin (TTX) abolished all responses to electrical stimulation, demonstrating their neural origin. Vertical scale indicates maximal response to KCl (160 mM). Horizontal arrowheads show the height of the primary tonic contraction recorded in the presence of atropine and apamin which has been used for quantitative evaluation. Asterisks mark the 'off' contraction.

Figure 2 Typical tracing illustrating the effect of apamin on spontaneous activity of circular muscle strips from the guinea-pig ileum, unmasking of an atropine-resistant primary contraction to electrical stimulation (5 Hz) and its inhibition by MEN 10,376 (15 min before-hand) an NK₂ receptor-selective tachykinin antagonist. Vertical scale indicates maximal response to KCl (160 mM). Asterisks mark the 'off' contractions.

consisted of the following phases: (1) initial twitch-like contraction, starting 1–2 s after the beginning of stimulation; (2) tonic contraction with superimposed phasic contractions; this phase could be clearly distinguished from the initial response and was present until stimulation was stopped; (3) 'Off' contraction upon cessation of stimulation, followed by a variable number of phasic contractions while the intercontraction tone was returning to baseline. All these components of the motor response were abolished by tetrodotoxin (Figure 1).

For quantitative evaluation, the peak of the tonic component (phase 2) of the contractile response was used (see Figure 1) because of its highest sensitivity to TK antagonists. Phase 3 was considerably less affected by TK antagonists while phase 1, which was depressed similarly to the tonic contraction, was more variable during repeated control stimulations.

For the rest of the study of atropine-resistant contraction, a stimulation frequency of 5 Hz was selected in order to produce a tonic primary contraction averaging 30–40% of maximal response to 160 mM KCl.

The effect of GR 71,251, MEN 10,376 and L 659,877 on the response to electrical stimulation (5 Hz for 30 s) was tested in the presence of atropine and apamin. GR 71,251 (10 μ M) produced 52% inhibition of the evoked response (Figure 3). MEN 10,376 caused 47 and 75% inhibition of the evoked response at 3 and 10 μ M, respectively (Figure 3, see also Figure 2). L 659,877 failed to elicit a significant change at all concentrations tested (Figure 3).

Figure 3 Effect of (a) GR 71,251 (b) MEN 10,376 or (c) L659,877 on the primary tonic contraction produced by electrical stimulation of circular muscle strips from the guinea-pig ileum in the presence of atropine (2 μ M) and apamin (0.1 μ M). For each panel, the control (Con) response is compared to that obtained in the presence of the antagonist. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P < 0.05$.

Circular muscle strips: effect of antagonists on the response to receptor selective tachykinin agonists

From the above experiments, GR 71,251 and MEN 10,376 were selected for further evaluation of selectivity in blocking tachykinin receptors.

To check the selectivity of antagonists for NK₁ vs NK₂ TK receptors, circular muscle strips were challenged at 20 min intervals with the NK₁ receptor-selective agonist [Sar⁹] substance P sulphone or the NK₂ receptor-selective agonist [β Ala⁸] neurokinin A (4–10) (300 nM for each agonist) which resulted in contractions of similar size (30–50% of the maximum) to those evoked by electrical stimulation at 5 Hz. Here again, the responses consisted of a tonic contraction and superimposed phasic contractions. The amplitude of tonic contractions was measured to quantitate the effect of antagonists. These experiments were performed in the presence of atropine and apamin.

Data shown in Figure 4 confirmed the selectivity of GR 71,251 and MEN 10,376: GR 71,251 (10 μ M) reduced (61% inhibition) the response to the NK₁ receptor-selective agonist, while leaving the response to [β Ala⁸] neurokinin A(4–10) unaffected. By contrast, MEN 10,376 did not affect the response to [Sar⁹] substance P sulphone while reducing (80% inhibition) that to the NK₂ receptor-selective agonist.

Figure 4 Effect of (a) GR 71,251 or (b) MEN 10,376 on the tonic response of circular muscle strips from the guinea-pig ileum to the NK₁ tachykinin receptor-selective agonist [Sar⁹]-SP sulphone or the NK₂ receptor-selective agonist [βAla⁸]-NKA(4-10) in the presence of atropine and apamin. For each panel, the control (Con) response is compared to that obtained in the presence of the antagonist. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P < 0.05$.

Effect of antagonists on the response to field stimulation in the absence of atropine and apamin

The effect of GR 71,251 and MEN 10,376 was also investigated on the primary contraction produced by electrical stimulation in the absence of atropine and apamin. A frequency of 10 Hz (other parameters as described above for the atropine-resistant response) was selected to obtain consistently a tonic contraction comparable to that produced by 5 Hz in the presence of atropine and apamin. Data shown in Figure 5 indicate that GR 71,251 (10 μM) or MEN 10,376 (3 μM) had no effect, while 10 μM MEN 10,376 produced a significant reduction of the evoked response.

Ascending enteric reflex

Distension of the balloon (0.1 ml) in untreated preparations produced a rapid contraction of the ileum ($84 \pm 5\%$ of maximal response to KCl, $n = 15$) which was largely or totally atropine-sensitive (reduction 85% or more), confirming previous observations from Costa *et al.* (1985b) and Holzer (1989). In the presence of atropine (2 μM) an atropine-resistant response to distension developed in about 30 min, the amplitude of which averaged $61 \pm 5\%$ ($n = 16$) of the maximal contraction to KCl. The atropine-resistant reflex required larger distension of the balloon (0.2–0.3 ml) than that produced in the absence of atropine to give a maximal response. In confirmation of previous observations (Holzer *et al.*, 1989), apamin (0.1 μM) enhanced the amplitude of the atropine-resistant response to distension, which averaged $81 \pm 4\%$ of the KCl response ($n = 19$).

The effect of GR 71,251 (10 μM), MEN 10,376 (3 and 10 μM) and L 659,877 (10 μM) was first studied in the presence of atropine and apamin, in order to match experiments performed in circular strips. In these experiments, MEN 10,376 had a limited and statistically not significant effect at 3 μM, while producing a significant inhibition at 10 μM (about 50% reduction as compared to control, Figure 6). GR 71,251 or L 659,877 (10 μM, $n = 4$ for each peptide) failed to affect the ascending enteric reflex in the presence of atropine and apamin (Figure 6).

The effect of MEN 10,376 (3–10 μM) was also investigated in the absence and presence of atropine (balloon distension with 0.1 and 0.3 ml, respectively, Figures 7 and 8). Apamin was not present in these experiments. Data in Figure 8 show that 3 μM MEN 10,376 had no effect in the absence of atropine while 10 μM significantly reduced the evoked response by about 30%. In the presence of atropine, both

Figure 5 Effect of GR 71,251 or MEN 10,376 on the tonic contractile response of circular muscle strips from the guinea-pig ileum to electrical field stimulation (10 Hz) in the absence of atropine. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P < 0.05$.

Figure 6 Effect of (a) GR 71,251, (b) MEN 10,376 or (c) L 659,877 on the ascending excitatory reflex in the guinea-pig isolated ileum elicited by balloon distension in the presence of atropine and apamin. Numbers in the columns show the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (80 mM). *Significantly different from controls: $P < 0.05$.

concentrations of MEN 10,376 were effective and the response was inhibited by 39 and 70% at 3 and 10 μM, respectively. In the presence of atropine, complete inhibition of the evoked response by 10 μM MEN 10,376 was observed in 3 out of 10 cases tested.

Discussion

Previous studies have implicated TKs as transmitters in the atropine-resistant contraction of the circular muscle of the guinea-pig ileum produced by electrical field stimulation or stimulation of the ascending enteric reflex (Costa *et al.*, 1985a,b; Holzer, 1989). The aim of this study was to gain

Figure 7 Typical tracing showing the effect of MEN 10,376 (contact time 15 min) on the ascending enteric reflex of the guinea-pig isolated ileum in the absence (upper panel) or presence (lower panel) of atropine. Vertical scale indicates maximal response to KCl (80 mM).

Figure 8 Effect of MEN 10,376 on the ascending enteric reflex of the guinea-pig isolated ileum in the absence (a) or presence (b) of atropine. Numbers in the columns show the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (80 mM). Con = control. *Significantly different from controls: $P < 0.05$.

information about the relative role of NK_1 and NK_2 tachykinin receptors in mediating circular muscle contraction produced by activation of noncholinergic nerves in the ileum. Assuming a concomitant release of SP and NKA from tachykininergic nerves of the ileum and, owing to the preferential activity of SP at NK_1 and NKA at NK_2 receptors, respectively, the use of receptor-selective TK antagonists should theoretically yield information about the relative contributions of different TKs to the overall response (cf. Maggi *et al.*, 1991b,c). The conclusions of this study are strictly dependent on the selectivity of the antagonists used.

GR 71,251 is a newly developed derivative of substance P which acts as a competitive NK_1 receptor antagonist having at least 100 fold selectivity over NK_2 or NK_3 receptors. In the longitudinal muscle of the ileum, and with substance P methylester as an agonist, GR 71,251 reportedly blocks NK_1

receptor activation with a pA_2 value of 7.7–7.8 (Hagan *et al.*, 1990; Hall & Morton, 1991).

MEN 10,376 and L 659,877 have been characterized as NK_2 receptor selective antagonists having at least 100 fold selectivity over NK_1 or NK_3 receptors (Williams *et al.*, 1988; Maggi *et al.*, 1991c). For both peptides, however, affinity at NK_2 receptors varies in different preparations and this has been taken as evidence for the existence of NK_2 receptor subtypes (Maggi *et al.*, 1990c; 1991d; Van Giersbergen *et al.*, 1991). In the present study, GR 71,251 and MEN 10,376 behaved as selective NK_1 and NK_2 receptor antagonists, respectively. L 659,877 failed to affect significantly the atropine-resistant response to nerve stimulation. This may indicate the presence, in the circular muscle of guinea-pig ileum, of an NK_2 receptor subtype for which L 659,877 has low affinity (or anyway lower affinity than that of MEN 10,376).

The reason(s) why a TK-mediated response became evident only in the presence of apamin cannot be conclusively determined on the basis of present findings. Whatever the mechanisms involved, an atropine-resistant circular muscle contraction sensitive to TK antagonists is easily detected by use of the ascending reflex arrangement, even without addition of apamin. It is evident that in the latter case, a discrete intramural neuronal circuitry is activated, leading to excitation of circular muscle at the recording site (Tonini & Costa, 1990), while electrical field stimulation is likely to activate all intramural neurones.

As far as the participation of NK_1 receptors is concerned, GR 71,251, at a concentration that selectively blocks NK_1 receptors in the ileum, reduced the noncholinergic response of circular strips to field stimulation while it had almost no effect on the atropine-resistant ascending enteric reflex. In both cases, experiments were performed in the presence of apamin. The available data indicate that although NK_1 receptors are present in the circular muscle of the ileum they play only a minor role during its physiological activation. Owing to the limited supply of GR 71,251 we were unable to extend the analysis of its action to other experimental conditions (e.g. studying the atropine-resistant ascending reflex in the absence of apamin).

The inhibitory actions of MEN 10,376 (3–10 μ M) on the ascending enteric reflex and on the electrically-evoked circular muscle contractions provide strong experimental evidence for an involvement of NK_2 receptors both in the physiological and in electrically-evoked activation of the circular muscle of the ileum by endogenous TKs. MEN 10,376, at concentrations (3–10 μ M) that selectively block NK_2 receptors in the circular muscle, inhibited the atropine-resistant ascending enteric reflex both in the absence and presence of apamin, as well as the primary contraction of circular muscle strips to electrical stimulation. MEN 10,376 also reduced the ascending enteric reflex and the response to electrical field stimulation in the absence of atropine, by about 30%. A similar effect has been reported previously when a high concentration (30 μ M) of Spantide was used (Holzer, 1989) and may indicate a partial involvement of TKs in the ascending enteric reflex evoked in the absence of atropine. Confirming previous observations (Holzer, 1989) larger volumes of balloon distension were required to evoke maximal noncholinergic circular muscle contractions than those effective without atropine. This agrees with the hypothesis (Costa *et al.*, 1985a; Grider, 1989) that the tachykininergic component of the ascending reflex comes into action at larger degrees of radial stretch than the cholinergic one.

In conclusion, a major role for NKA, or anyway for endogenous ligands with high affinity for NK_2 TK receptors, can be postulated in the excitatory response of the circular muscle of the guinea-pig ileum to nerve stimulation.

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