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Effects of imidazoline derivatives on cholinergic motility in guinea-pig ileum: involvement of presynaptic α_2-adrenoceptors or imidazoline receptors?

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Abstract The present study investigates the possibility that imidazoline receptors mediate modulation of cholinergic motor functions of the guinea-pig ileum. For this purpose, the effects of a series of compounds with known affinity for α_2-adrenoceptors and/or imidazoline recognition sites were examined on the cholinergic twitch contractions evoked by electrical field stimulation (0.1 Hz) of longitudinal muscle-myenteric plexus preparations. Additional experiments were carried out on ileal strips preincubated with [^{3}H]choline, superfused with physiological salt solution containing hemicholinium-3, and subjected to electrical field stimulation (1 Hz). The stimulation-induced outflow of radioactivity was taken as an index of endogenous acetylcholine release.

α-Methyl-noradrenaline, noradrenaline, clonidine, medetomidine, oxymetazoline and xylazine caused a concentration-dependent inhibition of twitch responses (IC_{50} from 0.13 to 1.05 μM; E_{max} from 85.9 to 92.5%). Rilmenidine and agmatine were less potent in reducing the twitch activity, and the latter compound acted also with low intrinsic activity (IC_{50}=44.9 μM; E_{max}=35.5%). In interaction experiments, the inhibitory action of clonidine on twitch responses was competitively antagonized by RX 821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline), idazoxan, rauwolscine, yohimbine and BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)-methyl]-4,5-dihydroimidazoline), whereas prazosin (10 μM), ARC 239 (2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione; 10 μM) and BRL 41992 (1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazol[1,5-a]azepine; 10 μM) were without effect. Rauwolscine antagonized the inhibitory effects of various agonists on ileal twitch activity in a competitive manner and with similar potency. Agmatine and idazoxan did not significantly modify the twitch contractions when tested in the presence of α_2-adrenoceptor blockade by rauwolscine (3 μM) or RX 821002 (1 μM). Linear regression analysis showed that the affinity values of antagonists correlated with their affinity at the α_{2A} and α_{2D} binding sites as well as at previously classified $\alpha_{2A/D}$ adrenoceptor subtypes, whereas no significant correlation was obtained when comparing the potency estimates of agonists and antagonists with the affinity at I_1 or I_2 binding sites. When tested on the electrically induced outflow of tritium, α-methyl-noradrenaline, noradrenaline, clonidine, medetomidine, oxymetazoline, xylazine and rilmenidine yielded inhibitory concentration-response curves which were shifted rightward to a similar extent in the presence of rauwolscine (3 μM). In the absence of further drugs, agmatine significantly reduced the evoked tritium outflow at the highest concentrations tested (10 and 100 μM), whereas idazoxan (up to 100 μM) was without effect. When RX 821002 (1 μM) was added to the superfusion medium, neither agmatine nor idazoxan modified the evoked outflow of radioactivity.

The results argue against modulation by imidazoline receptors of acetylcholine release from myenteric plexus nerve terminals. They provide evidence that compounds endowed with imidazoline-like structures affect the cholinergic motor activity of the guinea-pig ileum by interacting with presynaptic α_2-adrenoceptors belonging to the α_{2D} subtype.

Key words Imidazoline receptors · α_2-Adrenoceptors · Presynaptic receptors · Cholinergic transmission · Acetylcholine release · Intestinal motility · Isolated ileum

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Introduction

Several reports suggest that the effects of drugs such as clonidine, previously believed to depend on their activity at α_2-adrenoceptors, may indeed be due to their interaction with a novel family of non-adrenergic receptors named imidazoline receptors (Regunathan and Reis 1996). The pharmacological profile of imidazoline receptors consists mainly of very low affinity for catecholamine derivatives and

preferential affinity for ligands endowed with imidazoline, imidazolidine or guanidine structures (Ernsberger et al. 1995; Regunathan and Reis 1996).

According to binding experiments, the imidazoline receptor family appears to include two distinct receptor subtypes, designated as I_1 and I_2 (Regunathan and Reis 1996). I_1 binding sites display high affinity for imidazolidines (clonidine, p-amino-clonidine), medium affinity for imidazolines (idazoxan, phentolamine), and low affinity for guanidines (aganodine, guanabenz; Ernsberger et al. 1995). I_2 binding sites are characterized by high affinity for idazoxan, guanabenz and cirazoline, and medium affinity for clonidine (Miralles et al. 1993). Further investigations have revealed the existence of additional imidazoline receptor subtypes, the pharmacological profile of which does not fit with that of I_1 or I_2 sites, and that have been temporarily named non-I_1-non-I_2 receptors (Molderings and Göthert 1995). In addition, Li et al. (1994) reported that agmatine, a decarboxylated arginine derivative, may behave as endogenous ligand for imidazoline binding sites.

It has been proposed that imidazoline receptors of the non-I_1-non-I_2 subtype may be located prejunctionally on axonal nerve terminals, where they may play a significant role in the modulation of transmitter release (Göthert and Molderings 1991; Molderings and Göthert 1995). Presynaptic imidazoline receptors mediating an inhibition of noradrenaline release have been characterized in heart, aorta, pulmonary artery and kidney of different mammalian species (Göthert and Molderings 1991; Molderings et al. 1991; Fuder and Schwarz 1993; Bohmann et al. 1994). In most of these studies, the presence of imidazoline receptors could be detected only after blockade of presynaptic α_2-adrenoceptors, suggesting the co-existence of both of these receptor sites on the same nerve terminals.

As far as the digestive tract is concerned, experiments performed with [^{3}H]clonidine or [^{3}H]idazoxan provided evidence for the presence of imidazoline binding sites in both gastric and intestinal tissues (Wikberg et al. 1991; Molderings et al. 1995). In addition, in vivo studies showed that agmatine stimulates gastric acid secretion and worsens experimental mucosal injury in rats (Glavin et al. 1995). However, the possible role played by imidazoline receptors in the regulation of intestinal motor functions and neurotransmitter release has not yet been investigated.

Cholinergic nerve terminals within the enteric nervous system are equipped with presynaptic α_2-adrenoceptors, the activation of which results in an inhibition of acetylcholine (ACh) release (Del Tacca et al. 1970, 1982). α_2-Adrenoceptors are heterogeneous (Bylund et al. 1994), and neurochemical studies performed on ileal preparations suggested that cholinergic neurotransmission is modulated by $\alpha_{2A/D}$-adrenoceptors (Blandizzi et al. 1993; Funk et al. 1995). In a previous report, Kapocsi et al. (1987) proposed that noradrenaline and xylazine (an imidazoline derivative) act at different sites on cholinergic nerve terminals of ileal Auerbach plexus. Subsequently, on the basis of indirect evidence, it was hypothesized that imidazoline receptors are not likely to play a significant role in the control of ACh release in the intestine (Blandizzi et al. 1993). However, this possibility has not been explored in detail. Overall, the present study was undertaken in order: 1) to assess whether there are imidazoline receptors, distinct from α_2-adrenoceptors, at cholinergic axon terminals in the guinea-pig ileum, the activation of which inhibits the release of ACh; 2) to gain further insight into the pharmacological profile of the α_2-adrenoceptor subtypes involved in the regulation of cholinergic transmission. For these purposes, affinity values estimated for imidazoline/α_2-adrenergic receptor ligands in the guinea-pig ileum, and those reported in binding and functional studies from the literature, were compared by means of correlation analysis.

Materials and methods

Animals. Male albino guinea-pigs, 300–350 body weight, were used throughout the study. They were fed standard laboratory chow and tap water ad libitum and were not used for at least 1 week after their delivery to the laboratory. The animals were housed, four in a cage, in temperature controlled rooms on a 12-hour light cycle at 22–24°C and 50–60% humidity. Their care and handling were in accordance with the provisions of the European Community Council Directive 86-609, recognized and adopted by the Italian Government.

Preparation. At the time of the experiment, the entire ileum was excised from the small intestine, with the exception of the distal 10 cm, and longitudinal muscle strips with Auerbach´s plexus attached were prepared according to the method of Paton and Vizi (1969).

Recording of twitch contractions. Longitudinal muscle strips, weighing 60–120 mg, were placed in Krebs solution aerated with 95% O_2+5% CO_2. The Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5 (pH 7.4±0.1). Ileal strips were set up in organ baths of 10 ml capacity (overflow system) at 37°C containing oxygenated Krebs solution, and connected vertically to isometric transducers (Basile, Comerio, Italy) under an initial resting tension of 1 g. The mechanical activity of the longitudinal muscle was recorded by a polygraph (Basile, Comerio, Italy). A pair of coaxial platinum electrodes were positioned at distance of 10 mm from the longitudinal axis of each preparation, and electrical field stimulation was delivered by means of a Grass S5 stimulator. Recurrent phasic contractions of the longitudinal muscle (twitch response) were evoked by square wave pulses (1 ms duration, 200–250 mA intensity) at a frequency of 0.1 Hz. This contractile activity was abolished by tetrodotoxin (1 μM) or atropine (0.01 μM), but was unaffected by hexamethonium (10 μM), indicating an involvement of postganglionic cholinergic nerves. The preparations were stimulated for 60 min and were washed four times at 15-min intervals before the beginning of experiments.

Agonists were added cumulatively to the bathing fluid in 0.5-log unit increments. A period of 3–5 min was allowed to elapse between subsequent increments of concentration in order to enable the full effect of the agonist to develop. Under the present experimental conditions, it was possible to obtain at least three concentration-response curves for a given agonist in the same preparation without significant desensitization, provided that a 60-min interval elapsed between two subsequent concentration-response curves. Because tissues recovered rapidly from maximally effective concentrations of agonists after washing, it was possible to examine the interaction of agonists and antagonists in the same preparation. For this purpose, 60 min were allowed to elapse between two consecutive concentration-response curves, the antagonist was added to the bath 20 min before agonist, and one of two ileal strips taken from each animal served as control and received the agonist alone in order to correct for possible time-dependent changes. Drugs were given in volumes ≤1% of total bath volume (10 ml).

Agonist potencies were expressed as IC_{50} (concentration required to produce 50% of the maximal inhibitory effect); the percent maximum inhibition of the control twitch response (E_{max}) was also evaluated. Both parameters were calculated from single concentration-response curves and then averaged. Antagonist potencies were estimated by means of Schild analysis (Arunlakshana and Schild 1959). All Schild plots were drawn by linear regression and statistical significance of differences between their calculated slopes and unity was tested under the null hypothesis (slope=1). If calculated slopes did not significantly differ from unity ($P>0.05$), the agonist-antagonist interaction was accepted as competitive in nature. After verifying that the slope of Schild plot did not differ significantly from unity, the slope was constrained to unity with consequent estimation of the antagonist potencies as pK_B values (Kenakin 1993; Jenkinson et al. 1995).

Measurement of the release of [^{3}H]acetylcholine. The measurement of the release of [^{3}H]ACh from isolated guinea-pig ileum was carried out according to the procedure previously described (Blandizzi et al. 1993). Longitudinal muscle strips of ileum, weighing 60–120 mg, were incubated in Krebs solution for 20 min and then loaded with methyl-[^{3}H]choline (3 µCi/ml) for 45 min in a 3-ml organ bath. Electrical field stimulation of 0.1-ms duration at 1 Hz was applied for 30 min during the loading period. At the end of the loading period, the strips were washed five times with Krebs solution, transferred to another organ bath (5-ml capacity) filled with Krebs solution and superfused at a flow rate of 1 ml/min with Krebs solution at 37°C, aerated with 95% O_2+5% CO_2. The superfusing Krebs solution contained 10 µM hemicholinium-3 in order to inhibit the reuptake of [^{3}H]choline liberated by the hydrolysis of the released [^{3}H]ACh. In some experiments, the superfusing solution contained also RX 821002 1 µM in order to block prejunctional α_2-adrenoceptors. The first 60-min collection of effluent was discarded (preperfusion), after which 3-min fractions were collected for 90 min. During the superfusion period the strips were stimulated for 3 min with square wave pulses (10 V/cm) of 1-ms duration at 1 Hz (180 pulses) in the 3rd (S_1) and 20th (S_2) collection periods. Test drugs were added to the superfusate in the 14th collection period. In agonists-antagonist interaction experiments the antagonist was added to the superfusate in the 9th collection period. Exposure to each drug continued until the end of the experiment.

The radioactivity of the fractions was determined by liquid scintillation counting (Betamatic, Kontron Instruments, Milan, Italy). At the end of each experiment the radioactive content of the strips was also determined. For this purpose, each strip was weighed and then incubated in 1 ml of 10% trichloroacetic acid at room temperature for 30 min. The supernatant (50 µl) was added to 5 ml of scintillator and the ^{3}H content of the tissue was measured by liquid scintillation spectrometry. It was previously demonstrated that the outflow of radioactivity from the strips in response to electrical stimulation is almost entirely due to labelled ACh (Vizi et al. 1984). The outflow of tritium was calculated as a fraction of the tritium content of the ileal strip at the onset of the respective collection period (fractional rate; min^{-1}). The increase in tritium outflow evoked by electrical stimulation was calculated as the difference ´total tritium outflow during the four collection periods subsequent to the onset of stimulation´ minus ´estimated basal outflow´. The basal outflow was estimated as mean value of the tritium efflux determined during four collection periods: two immediately before the onset of stimulation and two subsequent to the 12-min period in which the tritium outflow was increased by stimulation. The evoked outflow was then expressed as a percentage of the tritium content of the tissue at the onset of stimulation. The effect of test drugs on the evoked tritium outflow was expressed as ratio of the percentage release during the second (S_2) and the first (S_1) stimulation (S_2/S_1), namely in the presence and absence of drug respectively (Vizi et al. 1984; Trendelenburg et al. 1997). Antagonist potencies were expressed as pK_d values from the equation:
$K_d=[B]/(DR-1)$, where [B] is the molar concentration of the antagonist and DR is the ratio of equally effective concentrations of the agonist (IC_{50}) in the presence and absence of the antagonist (Furchgott 1972).

Drugs. The following drugs were used: oxymetazoline HCl, xylazine HCl, (–)noradrenaline bitartrate, prazosin HCl, atropine sulfate, tetrodotoxin, hemicholinium-3 bromide (Sigma, St. Louis, MO, U.S.A.); [R-[R,S](–)α-methyl-noradrenaline, RX 821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline HCl], (±)idazoxan HCl, rauwolscine HCl, yohimbine HCl, hexamethonium 2Cl, agmatine sulfate (RBI, Natick, MA, U.S.A.); rilmenidine dihydrogenphosphate (Servier, Paris, France); (±)medetomidine HCl (Farmos, Turku, Finland); clonidine HCl (Boehringer, Ingelheim, Germany); ARC 239 [2-(2,4-(O-methoxy-phenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione HCl] (kindly provided by Karl Thomae, Biberach, Germany); BRL 44408 [2-[2H-(1-methyl-1,3-dihydro-isoindole)methyl]-4,5-dihydroimidazoline] and BRL 41992 [1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazol[1,5-a]azepine HCl] (both kindly provided by SmithKline Beecham, Frythe, Welwyn, U.K.); methyl-[^{3}H]-choline chloride (80 Ci/mmol; Amersham Laboratories, Des Plaines, IL, U.S.A.).

Statistical analysis. Results are given as mean ±SEM. The significance of differences was evaluated by Student´s t-test. P values lower than 0.05 were considered to be significant; "*n*" indicates the number of experiments.

Results

Twitch contractions

In a first set of experiments, the effects of drugs with known affinity for either α_2-adrenoceptors or imidazoline receptors or both on cholinergic twitch contractions were examined. IC_{50} and E_{max} are listed in Table 1. α-Methyl-noradrenaline, noradrenaline, clonidine, medetomidine, oxymetazoline, and xylazine caused a concentration-dependent inhibition of twitch responses (IC_{50} range: from 0.13 to 1.05; E_{max} range: from 85.9 to 92.5%; Fig. 1A,B). Rilmenidine and agmatine decreased the twitch activity only at relatively high concentrations (Fig. 1B). In addition, agmatine acted with low intrinsic activity to reduce the electrically induced ileal contractions (Table 1).

In agonist-antagonist interaction studies, clonidine was used as the reference agonist, due to its good affinity for both α_2-adrenoceptors and imidazoline receptors. In the presence of different concentrations of RX 821002 (0.03,

Table 1 IC_{50} and E_{max} values of α_2-adrenoceptors and imidazoline receptor ligands with respect to inhibition of cholinergic twitch contractions evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips

Drug	IC_{50} (µM)	E_{max} (%)	*n*
α-CH_3-Noradrenaline	0.17 ± 0.03	92.5 ± 2.4	15
Noradrenaline	0.20 ± 0.04	89.2 ± 2.5	10
Clonidine	0.17 ± 0.04	87.8 ± 2.3	15
Medetomidine	1.05 ± 0.17	87.6 ± 2.5	12
Oxymetazoline	0.26 ± 0.11	85.9 ± 3.4	10
Xylazine	0.13 ± 0.04	86.4 ± 2.3	12
Rilmenidine	2.54 ± 0.51	78.7 ± 1.9	10
Agmatine	44.9 ± 4.3	35.5 ± 3.8	10

Values are reported as mean ±SEM.; "n" indicates the number of experiments

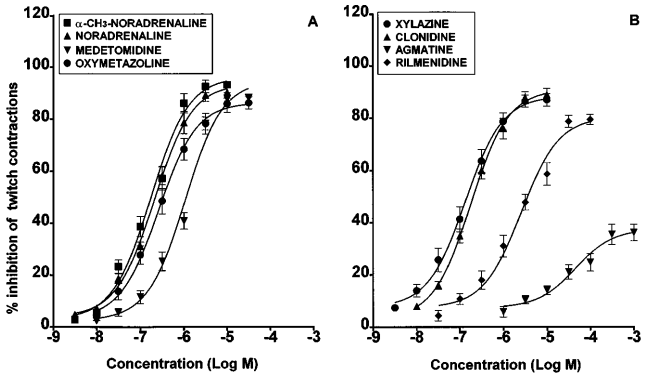

Fig. 1 Effects of α-methyl-noradrenaline, noradrenaline, medetomidine and oxymetazoline [A], xylazine, clonidine, rilmenidine and agmatine [B] on cholinergic twitch contractions evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips. Each concentration-response curve represents the mean of 10–15 experiments ±SEM

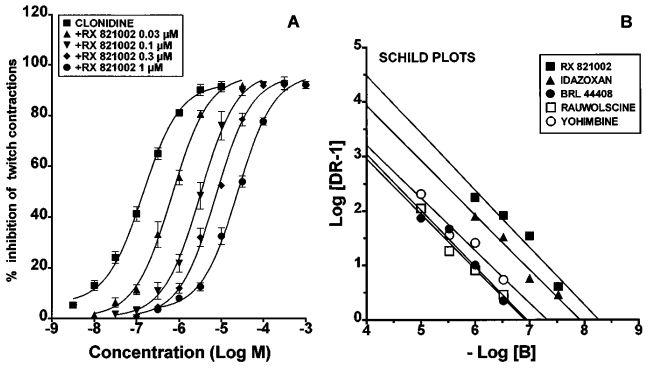

Fig. 2 A. Effect of clonidine on cholinergic twitch contractions evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips, in the absence and in the presence of increasing concentrations of RX 821002. B. Schild plots obtained for the antagonistic actions of RX 821002, idazoxan, BRL 44408, rauwolscine and yohimbine against the clonidine-induced inhibition of twitch activity. Each concentration-response curve represents the mean of 6–15 experiments ±SEM

Table 2 Antagonistic potency of α_2-adrenoceptor and imidazoline receptor ligands against clonidine-induced inhibition of cholinergic twitch contractions evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips

Drug	pK_B	Slope	P
RX 821002	8.34	–1.05	0.817
	(8.74–7.94)	(1.90–0.20)	
Idazoxan	7.92	–0.99	0.944
	(8.24–7.59)	(1.56–0.42)	
BRL 44408	7.01	–1.03	0.866
	(7.33–6.68)	(1.73–0.32)	
Yohimbine	7.26	–0.96	0.808
	(7.55–6.96)	(1.61–0.32)	
Rauwolscine	6.90	–1.02	0.874
	(7.22–6.58)	(1.52–0.50)	
Prazosin	<5.00	ND	ND
ARC 239	<5.00	ND	ND
BRL 41992	<5.00	ND	ND

Values reported for pK_B and slope, with 95% confidence limits in brackets, are the mean of a minimum of 6 determinations. Slope= slope of Schild plot. P= probability for slope of Schild plot to be significantly different from –1. ND= not determinable

Table 3 Antagonistic potency of rauwolscine against various agonists at presynaptic receptors mediating inhibition of cholinergic twitch contractions or tritium outflow ([^{3}H]ACh) evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips

Drug	pK_B	Slope	P	pK_d
α-CH_3-Noradrenaline	6.96	–1.08	0.653	7.05
	(7.29–6.63)	(1.74–0.42)		
Noradrenaline	7.11	–0.90	0.573	6.97
	(7.46–6.76)	(1.54–0.26)		
Clonidine	6.90	–1.02	0.874	6.95
	(7.22–6.58)	(1.52–0.52)		
Medetomodine	6.84	–1.15	0.396	6.91
	(7.20–6.48)	(1.75–0.55)		
Oxymetazoline	6.86	–0.92	0.700	6.98
	(7.25–6.47)	(1.69–0.15)		
Xylazine	7.01	–0.95	0.755	7.12
	(7.38–6.64)	(1.62–0.28)		
Rilmenidine	7.07	–0.98	0.891	7.15
	(7.42–6.72)	(1.56–0.40)		

Values reported for pK_B and slope, with 95% confidence limits in brackets, refer to twitch experiments and are the mean of a minimum of 6 determinations. Slope = slope of Schild plot. P= probability for slope of Schild plot to be significantly different from –1. Values reported for pK_d refer to [^{3}H]ACh release experiments and were determined from the rauwolscine-induced shift of the concentration-response curves (n=4 for each point) of the agonists at the level of IC_{50}

0.1, 0.3, 1 μM), idazoxan (0.03, 0.1, 0.3, 1 μM), BRL 44408 (0.3, 1, 3, 10 μM), yohimbine (0.3, 1, 3, 10 μM), or rauwolscine (0.3, 1, 3, 10 μM), there was a parallel displacement to the right of the concentration-response curve to clonidine. Fig. 2A shows the rightward shift of clonidine concentration-response curve obtained in the presence of RX 821002. Under the same conditions, prazosin, ARC 239 or BRL 41992, at concentrations up to 10 μM, failed to affect the inhibitory action of clonidine on cholinergic twitch contractions (not shown). Schild plots were constructed for each agonist-antagonist interaction (Fig. 2B), in order to calculate the respective slope and pK_B values (Table 2). Since Schild plot slopes were not significantly different from unity, the antagonist action was assumed to be competitive in nature.

Additional interaction experiments were performed in the presence of rauwolscine in order to assess whether each agonist acted at a common site of action in inhibiting the twitch responses. Rauwolscine (0.3, 1, 3, 10 μM) caused similar concentration-dependent displacements to the right in the concentration-response curves of α-methyl-norad-

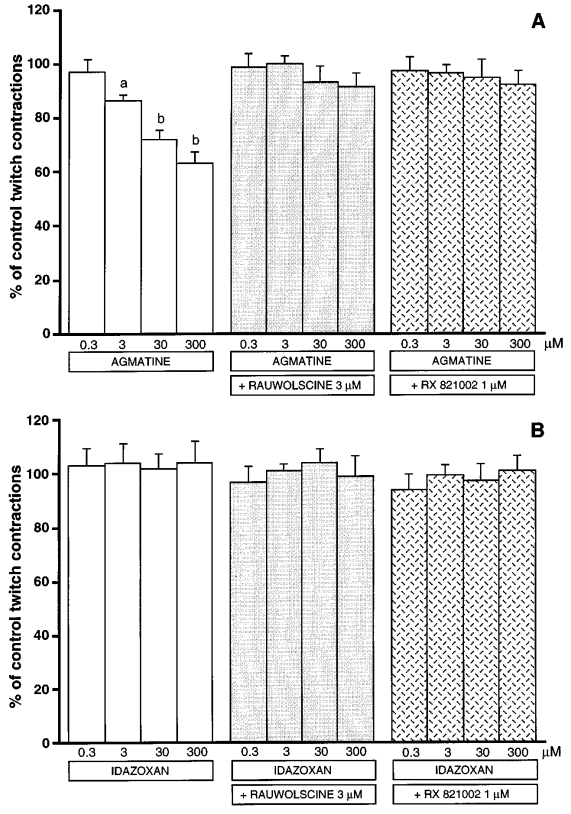

Fig. 3 Effects of agmatine [A] and idazoxan [B], tested either in the absence or in the presence of rauwolscine 3 μM or RX 821002 1 μM, on cholinergic twitch contractions evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips. Each column represents the mean of 4–6 experiments ±SEM. Significant difference from control values (100%): [a] $P<0.05$, [b] $P<0.01$

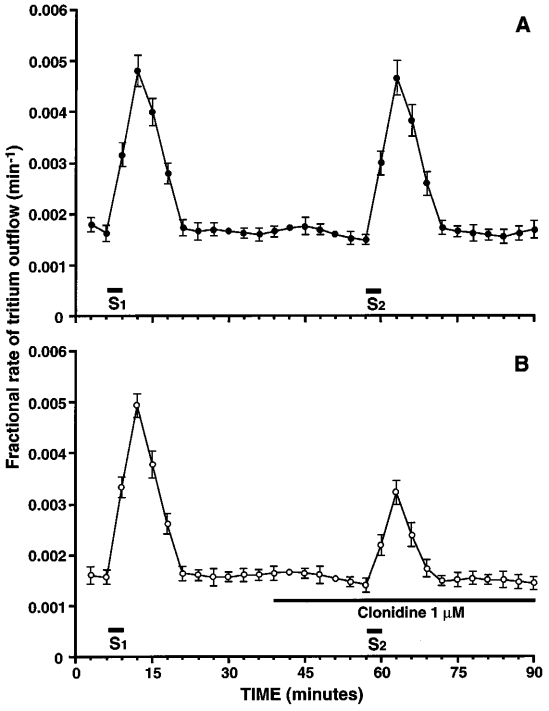

Fig. 4 Tritium efflux from guinea-pig ileum longitudinal muscle strips preincubated with methyl-[^{3}H]choline. Abscissa: time of superfusate collection. Ordinate: efflux of ^{3}H per min, expressed as fraction of tissue tritium at the onset of the respective collection period. Electrical field stimulation during S_1 and S_2 consisted of 180 pulses (10 V/cm, 1 ms) at 1 Hz. A Control experiments. B Effect of clonidine 1 μM present in the superfusion medium during the time indicated by the horizontal bar. Each point represents the mean of 4–6 experiments ±SEM

renaline, noradrenaline, clonidine, medetomidine, oxymetazoline, xylazine, and rilmenidine. Schild analysis indicated that rauwolscine acted in all cases as a competitive antagonist and that the respective pK_B values ranged from 6.84 to 7.11 (Table 3).

In a separate set of experiments agmatine (0.3–300 μM) or idazoxan (0.3–300 μM) were not able to significantly modify the twitch responses when applied to ileal preparations in the presence of rauwolscine 3 μM or RX 821002 1 μM (Fig. 3).

The correlation between potencies of drugs tested in the present study and pK_d values reported in the literature for the affinity of the same drugs to either native α_2 binding sites, α_2-adrenoceptor genes transfected into COS cells, previously classified presynaptic α_2-adrenoceptors, or native imidazoline binding sites, is shown in Tables 4 and 5. pK_B values significantly correlated with the pK_d values reported for native and recombinant α_{2A} and α_{2D} binding sites as well as for previously classified presynaptic $\alpha_{2A/D}$-adrenoceptor subtypes (Table 4), whereas no significant correlation was achieved when comparing pK_B and pIC_{50} values with pK_d values reported for I_1 or I_2 imidazoline binding sites (Table 5).

[^{3}H]Acetylcholine release

In control experiments, after a 60-min initial preperfusion period, the spontaneous tritium overflow approached a rate of 0.0018±0.00013 min^{-1} and remained unchanged throughout the experiment. When the superfused ileum strips were stimulated electrically, the tritium efflux increased significantly from 0.0016±0.00016 min^{-1} to 0.0048±0.0003 min^{-1} (n=6; $P<0.001$). The increase in tritium outflow evoked by electrical stimulation was observed usually in four consecutive 3-min fractions; the release reached a peak during this time, then declined exponentially to the prestimulation value. Under control conditions, the evoked tritium outflow was 2.39±0.26% for S_1 and 2.31±0.20% for S_2, not significantly different from each other (n=6); the calculated ratio S_2/S_1 was 0.97±0.05 (Fig. 4A). Fig. 4B shows the inhibition of the evoked tritium outflow obtained when S_2 was applied in the presence of clonidine 1 μM (S_2/S_1=0.42±0.04; n=4; $P<0.001$ versus control).

None of the agonists, assayed at concentrations ranging from 0.01 to 300 μM, modified the resting overflow of tritium either in the absence or in the presence of rauwolscine 3 μM (not shown). However, when tested on [^{3}H]ACh out-

Table 4 Correlation between affinity values (pK_B) obtained for the antagonistic effects of drugs against clonidine-induced inhibition of twitch contractions of guinea-pig ileum longitudinal muscle strips and pK_d values of the same drugs at prototypic α_2 binding sites or other presynaptic α_2-adrenoceptors

Binding sites	*r*	Slope	Number of compounds	P
Native α_2 binding sites				
Human platelets (α_{2A})[a,b]	0.883**	0.66	7	0.008
HT29 cells (α_{2A})[a]	0.919*	0.80	5	0.027
Neonatal rat lung (α_{2B})[a,b]	0.000	0.11	7	0.964
OK cells (α_{2C})[c]	0.837	0.58	5	0.076
Rat submaxillary gland (α_{2D})[b]	0.936**	0.72	7	0.002
RINm5F cells (α_{2D})[d]	0.705*	0.55	8	0.050
α_2 binding sites in COS cells transfected with human α_2 genes				
α_2-C10 (α_{2A})[e]	0.774*	0.61	7	0.041
α_2-C2 (α_{2B})[e]	–0.044	–0.03	7	0.930
α_2-C4 (α_{2C})[e]	0.427	0.21	7	0.338
α_2 binding sites in COS cells transfected with rat α_2 genes				
α_2-RNG (α_{2B})[f,g]	0.089	0.16	6	0.862
α_2-RG10 (α_{2C})[h]	0.728	0.39	4	0.271
α_2-RG20 (α_{2D})[f]	0.966**	0.65	5	0.007
Other presynaptic α_2-adrenoceptors				
Rat kidney autoreceptors ($\alpha_{2A/D}$)[i,k]	0.898**	0.39	7	0.006
Rat vas deferens autoreceptors ($\alpha_{2A/D}$)[l]	0.988**	0.62	5	0.002
Rat heart atria autoreceptors (α_{2D})[m,n]	0.927**	0.55	7	0.003
Rat vena cava autoreceptors (α_{2D})[n]	0.968**	0.66	5	0.007
Rat ileum heteroreceptors (α_{2D})[o]	0.952***	0.82	7	0.0009
Guinea-pig urethra autoreceptors(α_{2D})[n]	0.940**	0.75	6	0.005
Mouse heart atria autoreceptors (α_{2D})[p]	0.951*	0.56	5	0.013

Correlation coefficients (*r*) and slopes of the regressions "pK_d of ligands at prototypic α_2 binding sites" and "pK_d of ligands at other presynaptic α_2-adrenoceptors" on "pK_B estimated for antagonism of ligands against clonidine-induced inhibition of ileal twitch contractions". pK_B values are from Table 2 (4.5 for <5). pK_d values at prototypic α_2 binding sites or other presynaptic α_2-adrenoceptors are from studies quoted below. Significant difference from P: level of significance for deviation of r from zero

[a] Bylund et al. (1988);
[b] Renouard et al. (1994);
[c] Blaxall et al. (1991);
[d] Remaury and Paris (1992);
[e] Devedjian et al. (1994);
[f] Harrison et al. (1991);
[g] Xia et al. (1993);
[h] Lanier et al. (1991);
[i] Schwartz and Malik (1992);
[k] Bohmann et al. (1993);
[l] Smith and Docherty (1992);
[m] Limberger et al. (1992);
[n] Trendelenburg et al. (1997);
[o] Liu and Couper (1997);
[p] Wahl et al. (1996)

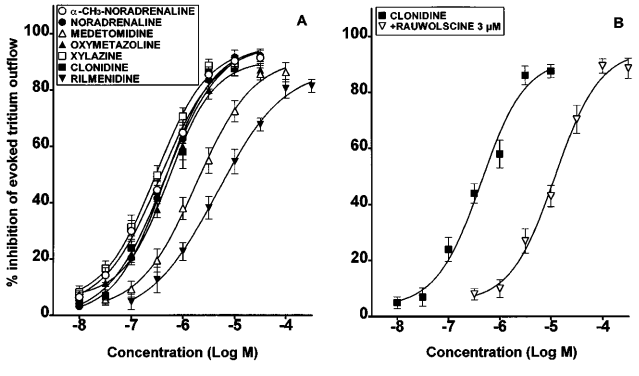

Fig. 5 Effects of α-methyl-noradrenaline, noradrenaline, medetomidine, oxymetazoline, xylazine, clonidine, rilmenidine [A], and clonidine either in the absence or in the presence of rauwolscine 3 μM [B] on outflow of tritium evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips preincubated with methyl-[^{3}H]choline. Each point represents the mean of 4 experiments ±SEM

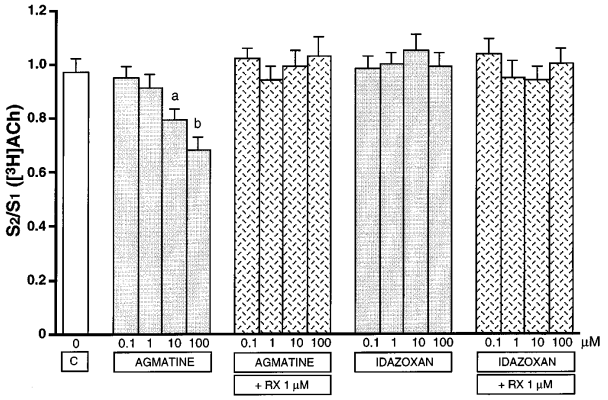

Fig. 6 Effects of agmatine and idazoxan, tested either in the absence or in the presence of RX 821002 1 μM (RX), on tritium outflow ([^{3}H]ACh) evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips. Each column represents the mean of 4–6 experiments ±SEM. Significant difference from control values (C): [a] P<0.05, [b] P<0.01

Table 5 Correlation between potency values (pIC_{50} or pK_B) estimated for the effects of drugs on cholinergic twitch contractions of guine-pig ileum longitudinal muscle strips and pK_d values of the same compounds at native imidazoline binding sites

Binding sites	*r*	Slope	Number of compounds	*P*
I_1 imidazoline binding sites				
Human reticularis lateralis[a]	0.256	0.69	5	0.679
Human platelets[b]	0.371	–0.61	8	0.365
I_2 imidazoline binding sites				
Human brain[c]	0.362	0.36	9	0.339
Rabbit cerebral cortex[d]	0.209	0.26	9	0.590
Guinea-pig cerebral cortex[e]	0.507	0.82	8	0.199
Rat brain[c]	0.308	0.33	9	0.421
Rabbit urethra[f]	0.352	0.52	7	0.438

Correlation coefficients (*r*) and slopes of the regressions "pK_d of ligands at imidazoline binding sites" on "pIC_{50} or pK_B for the effects of ligands on ileal twitch contractions." pIC_{50} or pK_B values are from Tables 1 and 2 (4.5 for <5), respectively. pK_d values at imidazoline binding sites are from studies quoted beside. *P*, level of significance for deviation of *r* from 0

[a] Bricca et al. (1989);
[b] Piletz et al. (1996);
[c] Miralles et al. (1993);
[d] Renouard et al. (1993);
[e] Wikberg an Uhlen (1990);
[f] Yablonsky et al. (1988)

flow evoked by electrical stimulation, α-methyl-noradrenaline, noradrenaline, clonidine, medetomidine, oxymetazoline, xylazine and rilmenidine yielded inhibitory concentration-response curves (Fig. 5A) which were shifted to the right in the presence of rauwolscine 3 μM, with pK_d values ranging from 6.91 to 7.15 (Table 3). Fig. 5B shows the rightward shift of clonidine concentration-response curve obtained in the presence of rauwolscine 3 μM.

Agmatine and idazoxan, assayed at concentrations ranging from 0.1 to 100 μM, did not significantly affect the resting overflow of tritium either in the absence or in the presence of RX 821002 1 μM (not shown). In the absence of RX 821002, agmatine (0.1–100 μM) significantly reduced the electrically induced outflow of [^{3}H]ACh only at the two highest concentrations tested, whereas idazoxan (0.1–100 μM) was without effect (Fig. 6). In the presence of RX 821002 1 μM, neither agmatine nor idazoxan modified the electrically induced release of [^{3}H]ACh (Fig. 6).

Discussion

The main purpose of the present study was to assess whether imidazoline receptors might modulate intestinal cholinergic neurotransmission. According to the present results, at least four lines of evidence suggest that this is not the case in the guinea-pig ileum. First, a variety of drugs with known α_2-adrenoceptor antagonist properties competitively blocked the inhibitory effect exerted by clonidine on the ileal twitch responses, irrespectively of whether they possessed imidazoline-like structure (RX 821002, idazoxan, BRL 44408) or not (yohimbine, rauwolscine). Second, rauwolscine, an α_2-adrenoceptor antagonist endowed with some affinity for prejunctional imidazoline receptors (Fuder and Schwarz 1993; Molderings and Göthert 1995), antagonized in a competitive manner and with similar potency the inhibitory effects of both imidazoline-like agonists (clonidine, medetomidine, oxymetazoline, xylazine, rilmenidine) and non-imidazoline agonists (α-methyl-noradrenaline, noradrenaline) on the twitch activity of ileal preparations. At variance with these findings, different pA_2 values were estimated for the antagonistic actions of rauwolscine against the inhibitory effects of several agonists (including clonidine and oxymetazoline) on electrically induced [^{3}H]noradrenaline release from rabbit aorta and pulmonary artery, indicating the existence of both prejunctional α_2-adrenoceptors and imidazoline receptors in these tissues (Molderings and Göthert 1995). Third, both agmatine and idazoxan failed to modify the twitch responses of ileal strips when tested in the presence of rauwolscine or RX 821002, which blocks with high affinity all α_2-adrenoceptor subtypes (pK_d from 8.8 to 9.7; O´Rourke et al. 1994; Renouard et al. 1994), but possesses no affinity for imidazoline receptors (pK_d<4.5; Miralles et al. 1993; Bohman et al. 1994). Fourth, the pIC_{50} and pK_B values estimated in the present study did not significantly correlate with the pK_d values reported in the literature for I_1 or I_2 binding sites. In this case, pIC_{50} values were tentatively used in addition to pK_B values for comparison with binding data since the agonist or antagonist properties of ligands for imidazoline sites have not been clearly established (Parini et al. 1996). It is also worth noting that the pIC_{50} value of agmatine (4.6), calculated for the present inhibitory action on cholinergic twitch contractions (Table 1), is consistent with pK_d values obtained in binding experiments at α_2-adrenoceptor subtypes (3.8–4.6; Piletz et al. 1995).

Further evidence against a modulation of cholinergic neurotransmission by imidazoline receptors came from our experiments showing that: (1) rauwolscine antagonized with similar potencies the inhibitory effects exerted by imidazoline-like or non-imidazoline agonists on electrically

induced [^{3}H]ACh release; (2) the stimulated tritium outflow was not affected by agmatine or idazoxan when tested in the presence of RX 821002. By contrast, previous reports indicated that imidazoline-like drugs (including clonidine and idazoxan) as well as agmatine inhibited [^{3}H]noradrenaline release from vascular sympathetic nerves in spite of α_2-adrenoceptor blockade, thus revealing the existence of prejunctional non-I_1-non-I_2 imidazoline receptors (Göthert and Molderings 1991; Molderings and Göthert 1995). Overall, it appears that, at variance with cardiovascular sympathetic nerve endings (Göthert and Molderings 1991; Fuder and Schwarz 1993), imidazoline-like compounds modulate cholinergic neurotransmission in the guinea-pig ileum only by interaction with presynaptic α_2-adrenoceptors. In support of this view, previous studies suggested that imidazoline receptors are not ubiquitously co-localized with α_2-adrenoceptors on prejunctional nerve terminals (Molderings and Göthert 1995).

In contrast to the present study, it was proposed that cholinergic nerve terminals of ileal Auerbach´s plexus are equipped with different noradrenaline- and xylazine- sensitive receptors, since yohimbine acted as a competitive antagonist against noradrenaline and as a non-competitive antagonist against xylazine (Kapocsi et al. 1987). However, other authors failed to confirm the findings of Kapocsi et al. (1987): yohimbine caused concentration-dependent rightward displacements of xylazine concentration-effect curves in the experiments of Akers et al. (1991); furthermore, in line with our findings, yohimbine antagonized in a competitive manner the inhibitory effects of clonidine on ileal twitch contractions (Drew 1978; Shebuski and Zimmerman 1985), thus indicating that in this preparation imidazoline derivatives interact with a homogeneous receptor population represented by α_2-adrenergic sites. It must be noted also that the IC_{50} values obtained in the present study for the inhibitory actions of noradrenaline and xylazine on twitch contractions (0.20 and 0.13 μM, respectively) were more than tenfold lower than those reported by Kapocsi et al. (1987; 2.5 and 3.7 μM, respectively). Although we could not reproduce the results of Kapocsi et al. (1987), our data are consistent with those of previous studies showing that xylazine inhibited the twitch activity of guinea-pig ileum with IC_{50} values ranging between 0.05 and 0.3 μM (Drew 1978; Akers et al. 1991).

In the present study, an attempt was also made to gain further insight into the pharmacological classification of α_2-adrenoceptor subtypes involved in the regulation of ileal cholinergic transmission. α_2-Adrenoceptors are pharmacologically distinguished in four subtypes, named α_{2A}, α_{2B}, α_{2C} and α_{2D}. However, three α_2-adrenoceptors have been cloned from human or rodent DNA libraries, and there is evidence to suggest that α_{2A} and α_{2D} sites represent species homologues of the same receptor subtype (see Bylund et al. 1994; Hieble et al. 1997).

Our experimental series on ileal twitch contractions showed that the inhibiting action of clonidine was counteracted by several α_2-antagonists, including BRL 44408 which discriminates between α_{2A}- and α_{2B}-adrenoceptors in binding experiments (Young et al. 1989). In addition, the inhibitory action of clonidine was not modified by prazosin, ARC 239 or BRL 41992, which possess preferential affinity for α_{2B}- or α_{2C}-adrenoceptors (Young et al. 1989; Bylund et al. 1994). According to the current classification criteria, the present data suggest that the drugs tested interacted with presynaptic α_2-adrenoceptors belonging to the $\alpha_{2A/D}$ subtype.

The comparative analysis between the pK_B values obtained for antagonists in the present study and the affinities of the same drugs for prototypic native and recombinant α_2 binding sites as well as for previously classified presynaptic α_2-adrenoceptors demonstrated a significant correlation with both α_{2A} and α_{2D} subtypes. This finding might suggest that α_2-adrenoceptors, located on cholinergic nerve terminals of guinea-pig ileum, possess pharmacological characteristics intermediate between those of human α_{2A} and rat α_{2D} subtypes. However, α_{2D}-adrenoceptors differ pharmacologically from α_{2A}-adrenoceptors mainly because of a lower affinity for yohimbine and rauwolscine (Bylund et al. 1994; Renouard et al. 1994). In this regard, it is interesting to note that the pK_B values obtained in our experiments for yohimbine (7.26) and rauwolscine (6.90) are more closely related to the affinities of these antagonists for α_{2D} (7.15 and 7.28, respectively) than for α_{2A} (8.50 and 8.72, respectively) binding sites (Renouard et al. 1994). In addition, the guinea-pig gene encoding the α_2-adrenoceptor homologue of the human α_2C10 subtype, has been recently cloned (Svensson et al. 1996), and yohimbine was found to bind to this receptor with a pK_d value of 7.68. Therefore, it appears that postganglionic cholinergic nerve terminals of myenteric plexus are equipped with α_{2D}-adrenoceptor subtypes. This view is in line with the conclusions of previous investigations, according to which the α_2-adrenoceptors involved in the prejunctional modulation of digestive cholinergic neurotransmission might be classified as $\alpha_{2A/D}$ subtypes (Blandizzi et al. 1993; Funk et al. 1995). In particular, it was initially proposed that α_2-adrenoceptors located on postganglionic cholinergic endings of guinea-pig ileum could belong to the α_{2A} or α_{2D} subtype (Blandizzi et al. 1993). Subsequently, Funk et al. (1995) provided more consistent evidence that ileal cholinergic terminals are equipped with α_{2D}-adrenoceptor subtypes.

In conclusion, the results obtained in the present study argue against an involvement of imidazoline receptors in the control of acetylcholine release from intestinal cholinergic nerves, and provide evidence that the compounds endowed with imidazoline-like structures can affect the cholinergic motor activity of guinea-pig ileum by interacting with presynaptic α_2-adrenoceptors belonging to the α_{2D}-subtype.

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