Guinea-pig ileum as *ex vivo* model useful to characterize ligands displaying Imidazoline I_2 and Adrenergic alpha$_2$ mixed activity: a preliminary study

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Abstract

The lack of an effective analgesic treatment makes pain a clinical challenge and the need of a novel approach to identify new agents is urgent. In this scenario I_2-ligands can be considered an alternative strategy in pain therapy. The development of an *ex vivo* model useful for the evaluation of functional activities at both α_2 and I_2-IBs (imidazoline binding sites) is an important task in pharmacological sciences since several I_2 ligands display activity also towards α receptors. The present study aims to develop an *ex vivo* model for estimating the activity of I_2-IBs ligands in a biological sample where α_1 and α_2 adrenergic receptors are present. For this purpose the imidalzoline endogenous ligand, harmane, reference compounds, 2BFI and BU224, and imidazoline derivatives 1-3 have been selected taking into account their *in vitro* activity towards IBs and adrenergic receptors. All compounds have been tested *ex vivo* in guinea pig-ileum where α_{2A}-ARs are prejunctionally and I_2-IBS postjunctionally localized. Adrenergic component has been identified by the studying the interference of compounds on the electrically-evoked contraction while I_2-IBs activity by testing the ability of compounds to inhibit the carbachol-evoked contractions in the presence of prazosin to mask the α_1 adrenoceptors. Compounds 1 and 2 were found I_2-IBs antagonists (pIC_{50}=4.2 and 4.0, respectively) whereas compound 3 was I_2-IBs agonist (EC_{50}=0.38 μM); All ligands were α_2 adrenergic agonists. This paper suggests guinea-pig ileum as the first ex vivo approach for establishing both the intrinsic activity of I_2-IBs ligands and the physiological correlation between IBs and adrenergic system.

Introduction

Imidazoline binding sites (IBs) are widely distributed in the central (CNS) and peripheral nervous systems (PNS) of mammalian cells.[1] Three IB subtypes have been proposed: I_1, I_2, I_3.[2-4] Among them, only I_1 subtype has been effectively characterized.[5] I_2-IB subtype, originally described as the Imidazoline-Guanidinium Receptive Site (IGRS) and characterized by idazoxan binding,[6] has been identified as allosteric binding site on monoamine oxidase (MAO) and on other non-MAO oxidative enzymes.[7] The interest about this I_2 subtype is due to its involvement in neuropathic and inflammatory pain.[8] Neuropathic pain is a condition often refractory to the classical pharmacological approach [opioids and non-steroidal anti-inflammatory drugs (NSAIDs)] that is treated by tricyclic antidepressants (TCAs), anticonvulsants and systemic local anesthetics. This treatment has a limited efficacy and several side effects.[8] The lack of an effective analgesic treatment makes pain a clinical challenge and the development of a novel approach is a real need. In this scenario I_2-ligands can be considered an alternative strategy in pain therapy. Indeed several evidences assessed the involvement of these sites in pain modulation: i) I_2 ligands are effective for tonic inflammatory and neuropathic pain less for acute phasic pain; ii) I_2 ligands in combination with opioids enhance their analgesic effects in acute phasic and chronic tonic pain; iii) I_2 ligands attenuate the development of tolerance to opioid analgesia.[8,9] All these evidences candidate I_2-IBs as targets for developing both potential pharmacological agents and diagnostic tools.

For this purpose, it is important to carry out a biological model useful to establish the activity of ligands towards I_2 sites considering the poor selectivity of the reported compounds towards the selected targets (I_2-IBs) due to the co-localization of α-adrenergic receptor (ARs) system. In fact, several I_2 ligands are also active towards ARs and consequently, their I_2-IBs and ARs contributions are difficult to be quantified in biological assay.

The aim of this paper is to develop an *ex vivo* method where the activity and the effects mediated by I_2-IBs could be directly identified and quantified taking into account in the meantime also the activity of I_2 ligands towards ARs.

Radioligand experiments demonstrated that I_2-IBs are present in high density in gastric and intestinal tissues but unfortunately, it was also reported the presence of α-adrenergic receptor subtypes in the same compartment.[10,11] IBs modulated cholinergic motor function in guinea-pig ileum through an interaction with presynaptic α_{2D}-adrenergic receptors.[12] This assumption was corroborated considering that the involvement of presynaptic imidazoline receptors in cholinergic motility of guinea pig ileum was excluded.[12] In addition, the involvement of α_1-ARs in mediating relaxation of the carbachol-evoked contraction in rat ileum longitudinal muscle has been demonstrated,[13,14] whereas an interaction between α_1-adrenergic receptors activation and I_2-IBs binding in the same organ has been hypothesized.[15]

Since some I_2 ligands can also display activities towards α_2 sites, we assessed as biological model guinea-pig ileum and as reference compounds ligands displaying: i) α_2 activity such as biphenyline (1) and its *m*-NO_2 analogue (2);[16] ii) mixed activity towards α_2 and I_2-IBs as cirazoline (3).[17] All these ligands have been already studied in medicinal chemistry reports both for their physical and in vitro biological properties.[16,17] The validation of this method has been performed also testing the proposed endogenous ligand such as harmane,[18,19] and known I_2 agonist and antagonist, 2BFI and BU224, respectively.[20,21]

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Materials and Methods

(*S*)-(–)-Biphenyline [(*S*)-(–)-1], (*R*)-(+)-*m*-NO_2-biphenyline [(*R*)-(+)-2] dihydrochloride and cirazoline (3) were synthesized according to published methods.[22-24] Clonidine, harmane, 2BFI and BU224 have been purchased by Tocris Bioscience, Ellisville, US.

Adult male Dunkin Hartley guinea-pigs (250-300g, Harlan Italy) were handled according to internationally accepted principles for care of laboratory animals (E.E.C. Council Directive 86/609, O.J. No. L358, December 18, 1986). The IC_{50} and EC_{50} values and SEM were obtained using non-linear curve fitting (Prism v. 3.0, GraphPad, San Diego, Calif., USA).

Isolated guinea pig ileum assay

Guinea pigs were anesthetized, decapitated and the proximal ileum removed. The intestine was carefully flushed several times with warm Krebs-Henseleit solution (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 0.6 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 11.2 mM glucose, pH 7.4) and placed in 20 mL organ bath containing Krebs-Henseleit solution at 37°C and bubbled with a 5% CO_2 and 95% O_2 gas. Krebs-Henseleit solution contained 1 μM indomethacin, 1 μM ketanserin, 1 μM pyrilamine, and 1 μM naloxone.

The strip (about 2 cm in length) was placed under 1.0 g load and tissue response, measured as changes in isometric tension, was recorded using a Fort 10 Original WPI isometric transducer (2 Biological Instruments, Milano, Italy) connected to a PowerLab/400 workstation (Ugo Basile, Milano, Italy). Each response is the mean (±S.E.M.) of at least three separate trials. Each experiment was repeated at least three times. Values of the concentration eliciting half-maximal contraction (EC_{50}) or half-maximal inhibition of contraction (IC_{50}) were determined by non-linear curve fitting (Prism v. 3.0, GraphPad).

α_2-AR agonist activity was measured as the ability to inhibit electrically evoked contraction of ileum.[25] The maximal effect in this assay has been previously determined in the presence of clonidine.

The strips were stimulated at 0.05 Hz using a digital stimulator (Letica 12106, Panlab, Besozzo VA, Italy) at 150 mA, 1 ms duration with platinum electrodes positioned longitudinally in the organ bath. Following a 90 to 120-min equilibrium period, during which Krebs solution was changed several times, test compounds were added as single dose (from 50 nM to 500 nM). The effectiveness of a given compound to inhibit electrically induced contraction was measured as the percentage change from baseline (Δg). The EC_{50} value was determined by plotting inhibition percentage *vs* logDose with a non-linear curve fitting program (Prism v. 3.0, GraphPad).

The contribution of postjunctionally localized imidazoline I_2-IBs was determined by testing the ability of compounds to inhibit the carbachol-evoked contractions in the presence of prazosin to mask the contribution of α_1 adrenoceptors.

The strip was stimulated with 200 nM carbachol that produced a tonic response. Compounds were tested as single dose ranging from 50 nM to 200 nM. The activity of compounds required to inhibit carbachol-induced contraction was measured as the percentage change from baseline (Δg) and the EC_{50} values were determined with a non-linear curve fitting program (Prism v. 3.0, GraphPad).

To study the contribution of I_2-IBS, 100 nM prazosin was added to the bath solution to mask α_1 adrenoceptors and the activity of tested compounds (concentration ranging from 50 nM to 200 nM) on carbachol-evoked contractions was measured. Agonist activity was measured as an EC_{50} value using the GraphPad program. Antagonist activity was determined by preincubating the tested compound for 15 min before adding the agonist and then evaluating carbachol response. The potency of antagonists was determined plotting the results in Schild analysis as pA_2 value. The pA_2 (-log [Antagonist]) was determined with a linear curve fit program (Prism v. 3.0, GraphPad) using the mean response of at least 3 separate trials.

Results

α_2-adrenergic receptors activity: effect on electrically-evoked contractions in guinea-pig ileum

In Figure 1A, compound 1, 2, and 3 displayed agonist effects below 300 nM with a comparable activity (EC_{50}=75 nM, 58 nM and 52 nM for compounds 1, 2, 3, respectively). At higher concentrations, higher than 300 nM, their effect was not dose-dependent and, as displayed in Figure 1B, all compounds seem to invert α_2-ARs activities.

In order to explain this dual activity, a molecular comparison study was carried out on biphenyline compounds. Some relevant pharmaceutical features of compounds 1-3 were calculated by means of QikProp molecular descriptors (QikProp, version 3.3, Schrödinger, LLC, New York, NY, 2010), and checked against similar properties for classical aminergic agonists such as adrenaline and noradrenaline (Figure 2). Interestingly, low similarity, as scored by the low values of the Tanimoto coefficient encoding for molecular surface (PSA, FOSA, SASA) and lipophilicity (QPlogo/w, QPlogBB), can be observed comparing adrenaline, and more dramatically noradrenaline to (*R*)-(+)-*m*-NO_2-biphenyline,[2] taken as reference. On the other hand, 1 and 3 showed similar figures to compound 2. This might suggest that, regardless of the presence of same pharmacophores, such as a cationic site and an aromatic ring spaced by a two atoms bridge, compounds 1, 2 and 3 do not share a common mechanism of action. As experimental evidence, passive permeability experiments, carried out using the PAMPA membrane method,[26] show that imidazolines can penetrate the cell membrane and could therefore act differently than catecholamine, which has low membrane permeability. These results indicate a different profile with respect to catecholamines for the compounds under investigation, and this could be ascribed to the presence, on the α_2-AR, of an allosteric binding site accessible to imidazolinic charged agonists and not aminergic. A similar hypothesis was proposed by Horstam and Ceresa,[27,28] who demonstrated an allosteric modulation by sodium ions, regulated by a negatively charged residue located in the second transmembrane helix of the α_2-AR.

I_2-imidazoline binding sites role on carbachol-evoked contractions in guinea-pig ileum

In order to elucidate the physiological role of

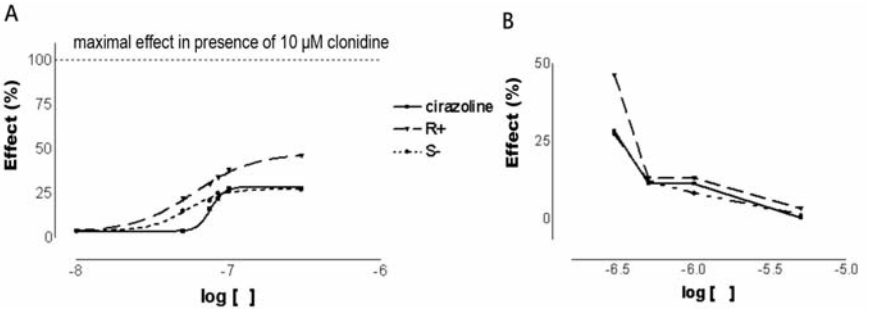


Figure 1. Representative dose-response curves of (R)-(+)-*m*-NO_2-biphenyline, (S)-(-)-biphenyline and cirazoline for α_2-adrenoceptors activity. This activity was observed below 300 nM for each compound in electrically-stimulated guinea-pig ileum (A). Representative dose-response curves at concentration >300 nM for each compound in the same experimental conditions (B). Statistical methods: one-way Anova (non parametric), P<0.0001, significant (P<0.05). Bartlett's test, P< 0.0001, significant (P< 0.05).

I_2-IBs, each compound has been studied towards contractile response induced by carbachol.[29] In this assay α_1-ARs were masked with 100 nM α_1 antagonist prazosin. The validation of this method has been performed testing the suggested endogenous I_2 ligand, harmane, and the putative agonist 2BFI and antagonist BU224. As expected (Figure 3), harmane inhibited the carbachol-evoked contraction in guinea-pig ileum (EC_{50}=8.3 μM) whereas the agonist 2BFI was partial agonist (α=0.7, EC_{50}=3.0 μM, respectively). The antagonist BU224 alone was inactive.

These agonists were tested in presence of I_2 antagonist BU224 (IC_{50}=1.0 μM) that displayed to be non competitive antagonist (Figure 4).

In vitro binding experiments showed that compound 1 weakly interacted with I_2-IBs whereas I_2-IBs profile of compound 2 was not studied.[16] Under the experimental conditions, without α_1 contribution, compounds 1 and 2 did not exert effects while compound 3 displayed potent inhibitory activity (EC_{50}=0.38 μM). Therefore, compound 3 could be considered I_2-IBs agonist. In addition, this effect was reversed by the I_2 antagonist Idazoxan (data not shown). The activity profile of compounds 1 and 2 was investigated studying their ability to reverse the effect of 3 as depicted in Figure 5.

Compound 1 and 2 seems to be I_2-IBs non competitive antagonists (pIC_{50}=4.2 and 4.0, respectively) with similar potency.

Discussion

In this work guinea-pig ileum was suggested as the first *ex vivo* approach for establishing both the intrinsic activity of I_2-IBs ligands and the physiological correlation between IBs and adrenergic system.

The development of an *ex vivo* model useful for the evaluation of functional activities at both α_2 and I_2-IBs is an important task in pharmacological sciences since to date selective I_2 ligands are not available since they display activity also towards α receptors.

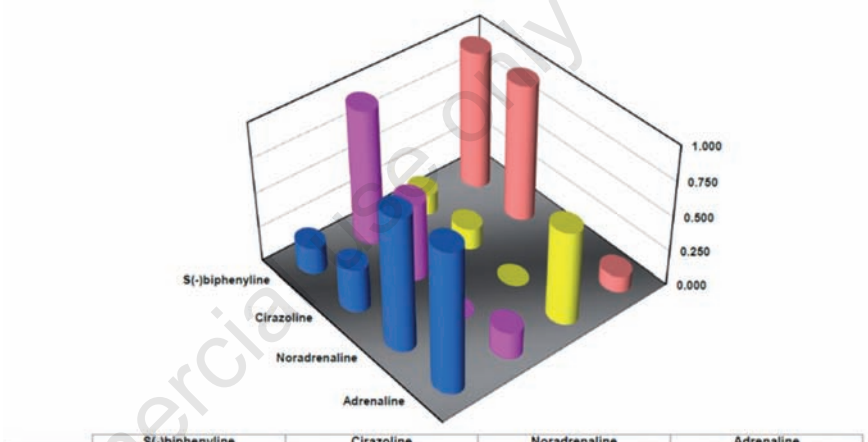
The interest about I_2 subtype is linked to its involvement in neuropathic and inflammatory pain.[8,9] The lack of an effective analgesic treatment makes pain a clinical challenge and the need of a novel approach to identify new agents is urgent. In this scenario I_2-ligands can be considered an alternative strategy in pain therapy.[8,9]

The aim of the present study is to assess the validity of guinea-pig ileum as *ex vivo* model to discriminate α_2 and I_2-IBs contribution of ligands exerting mixed activity. This goal could give a great contribution since these sites are important drug targets for treating pain and other diseases and can be considered as targets for the development of diagnostic tools.

In this *ex vivo* model the presence of α_{2A}-ARs, at prejunctional level, and I_2-IBS, at postjunctional level, is reported and this localization allows important piece of information about the physiological interaction of the studied ligands at the two sites.[10,12]

Each compound has been tested for its ability to inhibit electrically-evoked contractions in guinea-pig ileum in order to study α_2 receptors contribution. Indeed, α_2-AR agonists decrease the contractile response at prejunctional level. I_2-IBs activity has been evaluated testing the ability of all the studied compounds to inhibit the carbachol-evoked contraction in the same sample masking with prazosin α_1 sites. Indeed, I_2-IBs agonists decrease the contractile response at postjunctional level.[12,15]

For this purpose imidazoline derivatives 1-3 displaying mixed IBs and AR activity were selected. These ligands, tested for their ability to inhibit electrically evoked contractions in gunea-pig ileum, displayed agonist activity at α_2 receptors below 300 nM whereas at higher concentrations all compounds showed a decreased activity. Based on these results it could not be ruled out a possible receptor modulation elicited by these compounds, that the previous reported molecular similarity study,



	S(-)biphenyline	Cirazoline	Noradrenaline	Adrenaline
PSA	0.189	0.307	0.952	0.998
SASA	0.963	0.550	0.000	0.190
QPlogBB	0.146	0.149	0.000	0.664
QPlogPo/w	0.985	0.981	0.000	0.116

Figure 2. Tanimoto coefficients *versus* (R)-(+)-*m*-NO_2-biphenyline for the molecular features measured on α_2 agonists. PSA: Van der Waals surface area of polar nitrogen and oxygen atoms; FOSA: hydrophobic component of SASA; SASA: total solvent accessible surface using a probe with 1.4 Å radius; QPlogPo/w: predicted octanol/water partition coefficient; QPlogBB: predicted brain/blood partition coefficient.

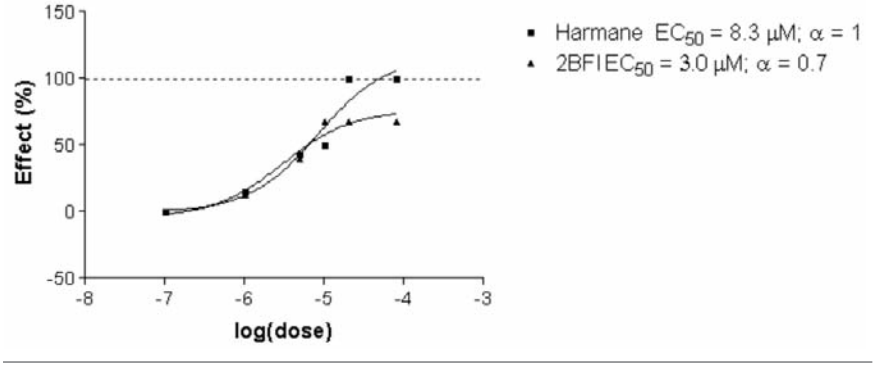


Figure 3. Representative dose-response curves of harmane and 2BFI on carbachol-mediated contractile response in guinea-pig ileum. P=0.001 (Wilcoxon test, P<0.05, significant). Statistical methods: one-way Anova (non parametric), P<0.0001, significant (P<0.05). Wilcoxon test, P=0.001, significant (P<0.05).

differentiated from classical α_2-AR ligands bearing catecholamines scaffold.

Moreover, since I_2-IBs modulated carbachol response at postjunctional level, for validating an useful *ex vivo* model to study I_2 activity of compounds exerting mixed I_2-α_2 profile, we tested as reference compounds the endogenous I_2-ligand, harmane,[18,19] and the putative I_2 agonist 2BFI and antagonist BU224.[20,21] The inhibition activity of reference compounds harmane and 2BFI have been quantified and their effects were partially reversed by the I_2 antagonist BU224. In the same experimental conditions, compound 3, reported as I_2-IBs putative agonist, together with compounds 1 and 2 were tested. In this assay, the contribution of I_2-IBs was evaluated by masking α_1-ARs contribution with the α_1-AR antagonist prazosin. Compound 3 displayed agonist activity while (*R*)-(+)-2 and (*S*)-(-)-1 derivatives were inactive. Moreover, the activity of compound 3 was reverted by the I_2-antagonist Idazoxan. The activity of (*R*)-(+)-2 and (*S*)-(-)-1 enantiomers was determined by their ability to reverse the effect of compound 3. Both ligands showed comparable antagonist activity towards I_2-IBs.

In conclusion, the present studies were aimed to the identification of *ex vivo* model that could be an useful tool in screening compounds and characterizing their agonist/antagonist activity at I_2-IBs and α_2-ARs. This method represents the first approach for establishing both the intrinsic activity of I_2-IBs ligands and the physiological correlation between IBs and adrenergic system.

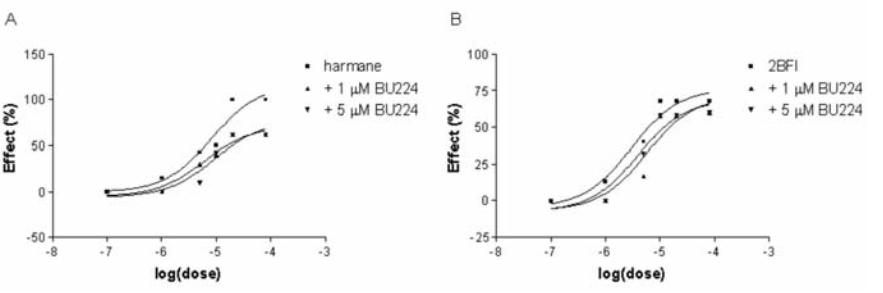


Figure 4. Representative dose-response curves of harmane and 2BFI in the absence and in the presence of at 1 or 5 μM BU224. Statistical methods: one-way Anova (non parametric), P<0.0001, significant (P< 0.05). Wilcoxon test, P= 0.001, significant (P<0.05).

Figure 5. Representative dose-response curves of cirazoline (I_2-agonist) on carbachol-mediated response in the absence and in the presence of (*R*)-(+)-*m*-NO_2-biphenyline and (S)-(–)-biphenyline (I_2-antagonist). Statistical methods: one-way Anova (non parametric), P<0.0001, significant (P<0.05). Bartlett's test, P<0.0001, significant (P<0.05)

Conclusions

The clinical relevance of this new *ex vivo* method is linked to the availability of a direct assay for screening potential drugs for the neuropathic and inflammatory treatment; this is an important task considering the urgent need of novel strategies because of the lack of an effective analgesic therapy. Since I_2-IBs are targets for potential pharmacological agents and diagnostic tools for pain therapy and several I_2 ligands displayed mixed activity towards I_2-IBs and α_2 adrenoceptors, the existence of a biological method to simultaneously study these two aspects could allow an efficacious *ex vivo* method. However, the limitation are due to the poor selectivity of some ligands towards I_2, α_2 and α_1 receptors that leads to employ masking compound in biological assay. Another limitation is the low number of ligands that could be screened by this method with respect to biochemical evaluation by cell lines overexpressing the specific binding site.

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