Guinea-pig ileum as *ex vivo* model useful to characterize ligands displaying Imidazoline I₂ and Adrenergic alpha₂ 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 I₁-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 \( I_2 \) and I₂-IBs (imidazoline binding sites) is an important task in pharmacological sciences since several I₂ ligands display activity also towards α receptors. The present study aims to develop an *ex vivo* model for estimating the activity of I₂-IBs ligands in a biological sample where \( I_1 \) and \( I_2 \) adrenergic receptors are present. For this purpose the imidazoline endogenous ligand, harmarane, 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 \( I_2 \)-ARs are prejunctionally and I₂-IBS postjunctionally localized. Adrenergic component has been identified by the studying the interference of compounds on the electrically-evoked contraction while I₂-IBS activity by testing the ability of compounds to inhibit the carbachol-evoked contractions in the presence of prazosin to mask the \( I_1 \) adrenoceptors. Compounds 1 and 2 were found I₂-IBs antagonists (pIC\(_{50}=4.2\) and 4.0, respectively) whereas compound 3 was I₂-IBs agonist (EC\(_{50}=0.38\) μM); All ligands were \( I_2 \) adrenergic agonists. This paper suggests guinea-pig ileum as the first *ex vivo* approach for establishing both the intrinsic activity of I₂-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 systems (PNS) of mammalian cells.¹ Three IB subtypes have been proposed: I₁, I₂, I₃.²-⁴ Among them, only I₁ subtype has been effectively characterized.³ I₂-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 I₂ 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.⁹ 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 I₂-ligands can be considered an alternative strategy in pain therapy. Indeed several evidences assessed the involvement of these sites in pain modulation: i) I₂ ligands are effective for tonic inflammatory and neuropathic pain less for acute phasic pain; ii) I₂ ligands in combination with opioids enhance their analgesic effects in acute phasic and chronic tonic pain; iii) I₂ ligands attenuate the development of tolerance to opioid analgesia.⁸,⁹ All these evidences candidate I₂-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 I₂ sites considering the poor selectivity of the reported compounds towards the selected targets (I₂-IBs) due to the co-localization of \( \alpha \)-adrenergic receptor (ARS) system. In fact, several I₂ ligands are also active towards ARs and consequently, their I₂-IBS and AR 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 I₂-IBs could be directly identified and quantified taking into account in the meantime also the activity of I₂ ligands towards ARs.

Radioligand experiments demonstrated that I₂-IBs are present in high density in gastric and intestinal tissues but unfortunately, it was also reported the presence of \( \alpha \)-adrenergic receptor subtypes in the same compartment.¹⁰,¹¹ IBs modulated cholinergic motor function in guinea-pig ileum through an interaction with presynaptic \( \alpha_2 \)-adrenergic receptors.¹² This assumption was corroborated considering that the involvement of presynaptic imidazoline receptors in cholinergic motility of guinea pig ileum was excluded.¹³ In addition, the involvement of \( \alpha_2 \)-ARs in mediating relaxation of the carbachol-evoked contraction in rat ileum longitudinal muscle has been demonstrated,¹⁴,¹⁵ whereas an interaction between \( \alpha_2 \)-adrenergic receptors activation and I₂-IBS binding in the same organ has been hypothesised.¹⁶

Since some I₂ ligands can also display activities towards \( \alpha_2 \) sites, we assessed as biological model guinea-pig ileum and as reference compounds ligands displaying: i) \( \alpha_2 \) activity such as biphenyl (1) and its \( m \)-NO₂ analogue (2);³⁶ ii) mixed activity towards \( \alpha_2 \) and I₂-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 harmaline,¹⁸,¹⁹ and known I₂ agonist and antagonist, 2BFI and BU224, respectively.²⁰,²¹

Materials and Methods

(S)-(−)-Biphenyline [(S)-(−)-1], (R)-(−)-m-NO₂-biphenyline [(R)-(−)-2] dihydrochloride and cirazoline (3) were synthesized according to published methods.²²-²⁴ Clonidine, harmaline, 2BFI and BU224 have been purchased by Tocris Bioscience, Ellisville, US.
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 EC50 values were determined with a non-linear curve fitting program (Prism v. 3.0, GraphPad).

To study the contribution of I2-IBS, 100 nM prazosin was added to the bath solution to mask α1 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 EC50 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 pA2 value. The pA2 (−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**

α2-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 (EC50=75 nM, 58 nM and 52 nM for compounds 1, 2, and 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 α2-ARs activities.

In order to explain this dual activity, a molecular comparison study was carried out on biphenyline compounds. Some relevant pharmacetical 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 (QPlogw, QPlogBB), can be observed comparing adrenaline, and more dramatically noradrenaline to (R)-(+)-(+)m-NO2-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 catecholamines, 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 α2-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 α2-AR.

**I2-imidazoline binding sites role on carbachol-evoked contractions in guinea-pig ileum**

In order to elucidate the physiological role of...
I2-IBs, each compound has been studied towards contractile response induced by carbachol. In this assay, each compound has been studied towards contractile response induced by carbachol. In this assay, α1-ARs were masked with 100 nM prazosin. The validation of this method has been performed testing the suggested endogenous I2 ligand, harmame, and the putative agonist 2BFI and antagonist BU224. As expected (Figure 3), harmame inhibited the carbachol-evoked contraction in guinea-pig ileum (EC50=8.3 μM) whereas the agonist 2BFI was partial agonist (α=0.7, EC50=3.0 μM, respectively). The antagonist BU224 alone was inactive.

These agonists were tested in presence of I2 antagonist BU224 (IC50=1.0 μM) that displayed to be non competitive antagonist (Figure 4).

In vitro binding experiments showed that compound 1 weakly interacted with I2-IBs whereas I2-IBs profile of compound 2 was not studied. Under the experimental conditions, without α1 contribution, compounds 1 and 2 did not exert effects while compound 3 displayed potent inhibitory activity (EC50=0.38 μM). Therefore, compound 3 could be considered I2-IBs agonist. In addition, this effect was reversed by the I2 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 I2-IBs non competitive antagonists (pIC50=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 I2-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 α2 and I2-IBs is an important task in pharmacological sciences since to date selective I2 ligands are not available since they display activity also towards α receptors.

The interest about I2 subtype is linked to its involvement in neuropathic and inflammatory pain. 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 I2-ligands can be considered an alternative strategy in pain therapy.

The aim of the present study is to assess the validity of guinea-pig ileum as ex vivo model to discriminate α2 and I2-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 α2ARs, at prejunctional level, and I2-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.

Each compound has been tested for its ability to inhibit electrically-evoked contractions in guinea-pig ileum in order to study α2 receptors contribution. Indeed, α2-AR agonists decrease the contractile response at prejunctional level. I2-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 α1 sites. Indeed, I2-IBs agonists decrease the contractile response at postjunctional level.

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 guinea-pig ileum, displayed agonist activity at α2 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.
differentiated from classical α2-AR ligands bearing catecholamines scaffold.

Moreover, since I2-IBs modulated carbachol response at postjunctional level, for validating an useful ex vivo model to study I2 activity of compounds exerting mixed I2-α2 profile, we tested as reference compounds the endogenous I2-ligand, harmaline,18,19 and the putative I2 agonist 2BFI and antagonist BU224.20,21 The inhibition activity of reference compounds harmaline and 2BFI have been quantified and their effects were partially reversed by the I2 antagonist BU224. In the same experimental conditions, compound 3, reported as I2-IBs putative agonist, together with compounds 1 and 2 were tested. In this assay, the contribution of I2-IBs was evaluated by masking α1-ARs contribution with the α1-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 I2-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 I2-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 I2-IBs and α2-ARs. This method represents the first approach for establishing both the intrinsic activity of I2-IBs ligands and the physiological correlation between IBs and adrenergic system.

**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 I2-IBs are targets for potential pharmacological agents and diagnostic tools for pain therapy and several I2 ligands displayed mixed activity towards I2-IBs and α2-ARs, 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 I2, α2 and α1 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.

**References**


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