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TESTING CONCEPTION OF ENGAGEMENT OF IMIDAZOLINE RECEPTORS IN IMIDAZOLINE DRUGS EFFECTS ON ISOLATED RAT HEART ATRIA

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Recently, attention has been payed to the role of imidazolines in physiology of the heart. However, no systematic comparative studies were reported regarding the activity of a representative set of specific ligands towards imidazoline receptors in the heart preparations. The aim of this project was to test effects of a set of ligands on the pharmacological function of putative imidazoline receptors in isolated rat heart atria. Known imidazoline drugs with a postulated high affinity to imidazoline I_1 receptor: AGN192403, rilmenidine, moxonidine and clonidine were used. The specific ligands of imidazoline I_2 receptor: 2-BFI, BU239 and putative natural ligand for imidazoline I_1, I_2 and I_3 receptors, agmatine, were tested also. The spontaneously beating right and left atria, driven electrically, were studied. Dose-response curves for amplitude and rate of the contractions of the atria were produced by administration of increasing doses of the agents. Phentolamine as α_1/α_2 adrenergic receptors blocker and idazoxan as I_2/I_1/α_2 receptors blocker were added in order to inhibit ino- and chronotropic effects of the compounds studied. The -log EC_{50} parameters were calculated. The positive inotropic effect on left atria were evoked with the rank order of potency: agmatine >> clonidine > BU239 > rilmenidine ≥ moxonidine and these effects were generally diminished by idazoxan. Moxonidine produced a weak positive inotropic effect potentiated by idazoxan. Rilmenidine and moxonidine were assumed to act as partial agonists of imidazoline I_1 receptor. AGN192403 did not change the amplitude of beating of left atria. The positive chronotropic effects on spontaneously beating right heart atria were with in the following order of potency: BU239 ≥ agmatine >>> clonidine > AGN192403. Idazoxan markedly antagonized chronotropic effect of both BU239 and agmatine. 2-BFI weakly diminished the rate of beating of atria; moxonidine and rilmenidine had no effect. In conclusion, imidazoline receptors of the I_1 subtype may be involved in inotropic reaction of the agents studied, but this effect depends mainly on the α_2/α_1 adrenergic receptors. Engagement of I_2 imidazoline receptors, along with the α_2 adrenergic ones, in chronotropic activity of isolated right atria of rat has been demonstrated.

Key words: imidazoline receptors, rat heart atria, chronotropic and inotropic effect

INTRODUCTION

Various imidazolines have cholinomimetic, sympathomimetic, histaminelike, antihistamine and adrenergic properties. Second generation central antihypertensives, such as moxonidine and rilmenidine, have attenuated sedative (α_2 adrenoceptor-mediated) side effects at equihypotensive doses compared with the drugs of first generation (1). While recognized as potent agonists at peripheral α_2 adrenergic receptors, several studies suggested that the drugs also had
Imidazoline receptors engaged in circulatory system are classified in two groups: the I\(_1\) type, sensitive to clonidine and idazoxan, an antagonist with an imidazoline structure, and the I\(_2\) type, displaying a high affinity for idazoxan (4), guanabenz (5, 6) cirazoline (7), and a medium-to-low affinity for clonidine (4).

Imidazoline I\(_1\) receptors are reported to play a role in the central regulation of blood pressure (8). The selective activation of central I\(_1\)-imidazoline receptors results in an inhibition of peripheral sympathetic activity and produces arterial vasodilatation (9, 10). Imidazoline agents evoke diverse pharmacological responses in both peripheral tissues and the central nervous system, so that are difficult to attribute to known receptor signaling system (11).

The presence of presynaptic imidazoline receptors has been suggested in the human and rat heart but their functional role is unknown (12). Imidazoline I\(_1\) receptors over \(\alpha_2\) adrenoceptors in the heart atria and ventricles have been identified with the affinity to imidazolines at nanomolar range (13). An 85 kD protein may correspond to the functional I\(_1\) receptor in atria. It has been shown that atrial I\(_1\) receptors are up-regulated in spontaneously hypertensive rats (SHR) (14, 15). El-Ayoubi et al. (14) observed that I\(_1\) receptors are increased in hypertensive rats or humans. Therefore, there are remisses to suppose the engagement of imidazoline receptors in ino- and chronotropism of isolated heart atria (16).

Functional cardiac imidazoline I\(_1\) receptors are tissue-specific, being differentially regulated in atria and ventricles in hypertension by chronic exposure to agonists (17).

In contrast to the I\(_1\) imidazoline receptors, physiological role for I\(_2\) sites has not yet been determined but it has been proposed that they play an active role in various physiological processes (18). For example, antiproliferative action of the imidazolines correlated with their affinity to the I\(_2\) imidazoline binding sites in blood vessels (2, 19). The imidazoline I\(_2\) binding sites (IBS) were described as imidazoline-guanidinium receptive site (IGRS) with idazoxan binding selectivity (20, 21). IBS appear to be heterogeneous in nature (22). Up today, their molecular structure, functional significance and their second-messenger system are unknown. I\(_2\) receptor ligands interact with a domain on MAO, a catecholamine metabolizing enzyme, but this mechanism is not equally accessible in all tissues (11, 23, 24).

Clonidine is twice as potent as moxonidine at the I\(_1\) receptor but has similar affinity for \(\alpha_2\) and \(\alpha_1\) adrenoceptors (25). In binding assay on cow brain the \(K_i\) values for clonidine at \(\alpha_2\) and I\(_1\) receptors were 3.8 and 1.0 nM, respectively (26). Hypotensive effect of clonidine is mainly through the \(\alpha_2\) adrenergic over I\(_1\) imidazoline receptors (27). Significant bradycardia of isolated rat heart was observed with clonidine, and less with moxonidine, at 10\(^{-5}\) M concentration. It suggests that postsynaptic cardiac imidazoline I\(_1\) receptors may be involved in these effects (28).

Moxonidine is pharmacologically similar to clonidine, but its affinity to imidazoline I\(_1\) receptors over \(\alpha_2\) adrenoceptors is 100-fold higher (29). The hypotensive mechanism of moxonidine originally suggested was through activation of central \(\alpha_2\) adrenoceptors, but it appeared that primary action in hypertension is due to binding of moxonidine at imidazoline I\(_1\) receptors in the rostroventromedial part of the brainstem (RVLM) (30). Moxonidine is three times more selective for the I\(_1\) receptor in RVLM than rilmenidine and has 40-70 times greater affinity for I\(_1\) receptors than for \(\alpha_2\) adrenoceptors (25). Moxonidine and rilmenidine injected intravenously lowered blood pressure, decreased plasma norepinephrine concentrations and inhibited stimulation-evoked cardioacceleration in pithed rabbits via \(\alpha_2\) adrenoceptor mechanism (31). Inhibition of norepinephrine release by moxonidine in pithed SHR was demonstrated by Raasch et al. (32): the authors explain this effect by interaction with imidazoline I\(_1\) receptor. Intravenous moxonidine may activate imidazoline I\(_1\) receptors and \(\alpha_2\) adrenoceptors present in the rat heart. Compared with clonidine selectivity, moxonidine and rilmenidine has approximately 3 to 10 times greater affinity for imidazoline receptors (14, 17).

Rilmenidine exhibited antiarrhythmic effects in different animal models of arrhythmia (33). This effect is likely to originate from effects on the central nervous system as well as from an action at peripheral sites (34).

Rilmenidine is neutral regarding metabolic parameters, but it influences left ventricular hypertrophy, microalbuminuria and insulin resistance positively in hypertensives at risk (35). Widimsky and Sirotiaková (36) observed decrease of arterial pressure and reduction in heart rate in hypertensive patients treated with 1mg rilmenidine daily. That may be a clinically relevant benefit in patients with an increased cardiovascular risk and metabolic disorders.
Agmatine (decarboxylated arginine) is widely distributed through the body, attaining high levels in the rat aorta (57.41 ng/g) and in the rat heart (6.03 ng/g), with the concentration in brain being relatively small (2.4 ng/g) (37). Molecular mass of agmatine is 130 Da (24). The agent was found to be regionally distributed in the rat brain (38). Agmatine is released from the neurons and interacts with various pre- and postsynaptic receptors including the I$_1$ imidazoline receptor, $\alpha_2$ adrenoceptor, NMDA receptor, nicotinic cholinergic receptor and 5-HT$_3$. receptor which might be of physiological importance (39, 40). All that suggests that agmatine may be a neurotransmitter (41). Their affinity (K$_i$) in human brain for $\alpha_{2A}$ and $I_1$ imidazoline receptors is 46 980 and 33.4 nM, respectively (26), although the interaction of agmatine with $\alpha_2$ adrenoceptors is unclear (42, 43). It has been postulated that agmatine may change uptake of norepinephrine, like clonidine does, thus reducing sympathetic tone through imidazoline receptors (42).

Agmatine concentration-dependently releases adrenaline and noradrenaline from chromaffin cells. This effect can be blocked by antagonists of the $I_1$ receptor. In chromaffin cells agmatine has a high affinity to $\alpha_2$ adrenoceptors and $I_1$ and $I_2$ binding sites, namely 4.0, 0.7 and 1.0 µM, respectively. It has a low affinity to the $\alpha_1$ and $\beta$ adrenoceptors as well as to the 5-HT$_1$, serotonin and D$_2$ dopamine binding sites. Agmatine-uptake into synaptosomes is blocked by the imidazoline derivative idazoxan and by phentolamine, but not by clonidine, moxonidine and rilmenidine (41).

Agmatine has no effect on vascular contraction and blood pressure, contrary to moxonidine, which increases the vascular contraction and decreases blood pressure (39). However, Herman (44) suggested that it can regulate cardiovascular functions and modulate some processes in the peripheral and central nervous system, whereas it has only a weak blood pressure effect when applied within the RVLM area. The affinity of agmatine for IBS is rather low, while it also binds $\alpha_2$ adrenoceptors (41). In the experiments on rat hearts, agmatine increased norepinephrine level indicating a synergistic inhibitory action at $I_1$ imidazoline receptors under conditions of stimulated $\alpha_2$ adrenergic autoinhibition (15).

Research on newly synthesized imidazoline compounds with a high affinity to imidazoline receptors is still carried on. It is believed that the binding sites specifically recognizing the imidazoline structure or similar chemical structures, both in the brain and in certain peripheral tissues, including the heart, participate in the control of blood pressure (14).

Imidazoline receptors bind some agents that are not imidazolines, such as guanabenz (45), guanidinium, rilmenidine (46) and oxazole. Newly synthesized analogues of imidazolines and reference oxazolines elicit an appreciably increased selectivity for $I_1$ and $I_2$ receptors. A few of these compounds, namely "AGN" and "BU families", show prevailing affinities for $I_1$ and $I_2$ receptors. These compounds are the first imidazoline analogues described that are devoid of any significant affinity to $\alpha_2$ adrenergic receptors.

The isofuran derivative of imidazoline, AGN 192403, is about 10,000 time more selective for imidazoline receptors than for $\alpha_2$ adrenoceptors (47). It had no effect on blood pressure when injected intravenously in monkeys and rabbits. AGN was found to be an antagonists of imidazoline receptor in a number of studies (15, 48).

The benzofuran derivative, 2-BFI, appeared 1,000 to 10,000 times more selective for [3H]idazoxan imidazoline specific binding sites than for $\alpha_2$ adrenoceptors. Selective for the $I_2$ imidazoline binding sites agent 2-BFI has $K_i$ of about 1 nM and a low activity for $\alpha_2$ adrenoceptors. It can elevate extracellular levels of norepinephrine in the frontal cortex and hippocampus in rats (49).

BU239 was described as a ligand with a high selectivity to $I_1$ receptors in rabbit brain. In competitive binding assays on rat kidney membranes $K_i$ for BU239 was 4.3 nM (16, 50).

Distinction between the imidazoline receptor and the $\alpha_2$ adrenoceptor-mediated mechanism for imidazoline compounds is difficult. A combination of agonists and antagonists differing in affinities for each receptor is required for that (26). The most frequently used imidazoline receptor antagonist, idazoxan, is also a potent $\alpha_2$ adrenoceptor antagonist. It seems that important for affinity of agents towards $I_1$ receptors is their hydrophobicity, as expressed by log P ranging from 1 to 2 (51). Therefore, structural alterations of idazoxan can result in molecules with a marked selectivity for either $\alpha_2$ adrenoceptors or imidazoline receptors.

Beside phentolamine, potent nonselective $\alpha_1$/$\alpha_2$ adrenoceptor antagonists with a low affinity at histaminergic receptors and less potent at imidazoline receptors are used. For now, there are no endogenous agonists selective for imidazoline receptors. All drugs binding to imidazoline receptors bind to $\alpha_2$ adrenergic receptors as well. It has not been possible to determine unambiguously whether drugs binding to $I$ receptors act as agonists or antagonists and have actions comparable to their actions at the $\alpha_2$ adrenergic receptors.

Imidazoline derivatives have been demonstrated to interact with the sympathetic neurotransmission via nonadrenergic presynaptic receptors in different
experimental models. However, no systematic comparative studies were reported regarding the inotropic and chronotropic activity of a representative set of imidazoline drugs towards imidazoline receptors in the heart preparations. On the other hand, single individual imidazolines have been reported to elicit pronounced pharmacological effects mediated through cardiac receptors. The direct effect of an imidazoline receptor ligand on cardiac receptors has not been established. Still, new imidazoline derivatives are potential drugs for antihypertensive therapy.

The aim of this study was to determine in vitro the inotropic and chronotropic effect of newly synthesized imidazoline receptor ligands: 2-BFI, BU239 and AGN192403 as well as the known imidazoline drugs, like clonidine, rilmenidine, moxonidine and agmatine on isolated rat atria. The agents studied were selected from the point of view of their hypothetical interaction with the adrenergic/imidazoline receptors in cardiac cells. The main task of this project was to help to direct rationally the further search for original circulatory and antihypertensive imidazoline agents based on their cardiotropic properties.

MATERIALS AND METHODS

The procedure applied was designed in accordance with the respective Polish and European regulations and the guidelines established by the Ethics Committee for Animal Experiments of the Medical University of Gdansk, Poland.

Materials

Male Wistar rats (200-350 g) were used for in vitro studies. The animals were housed and fed in a laboratory kept at constant temperature of 22°C under the standard conditions (12:12 h L:D cycle, standard pellet diet, tap water).

Methods

The animals were anesthetized with urethane (1.5 g/kg i.p.) and the heart was rapidly excised. After cervical dislocation, thorax was quickly opened, the still beating heart removed and placed in the preparation dish with a modified Krebs-Henseleit solution. The ventricular tissue was cut away as far as possible. The atria, left and right separately, were placed in the solution, gassed with 95 % O₂ and 5 % CO₂ giving pH of 7.3-7.4, and kept at 35.5-37°C. The incubation medium contained NaCl 118 mmol, KCl 4.7 mmol, CaCl₂ 6 mmol, NaH₂PO₄ 1 mmol, MgCl₂ 1.2 mmol, NaHCO₃ 25 mmol, glucose 11.1 mmol, EDTA 0.04 mmol, and ascorbic acid 0.1 mmol.

The muscle was tied at either end to stainless hooks under a tension of 0.5-1.0 g in an organ bath and was allowed to stabilize for 45-60 min. The left atrium was electrically stimulated with two platinum electrodes by square-wave electrical pulses (2.5 Hz, 4 ms) and voltage 10 V. Amplitude of contractile tension (mm) of the left atrium and the rate of contractile action (min⁻¹) of the spontaneously beating right atrium were recorded by means of an isometric force transducer (Bio-Sys-Tech, Bialystok, Poland).

Experimental protocol

After equilibration period, the cumulative concentration-response curves were constructed for increasing concentrations of the imidazolines studied ranging from 10⁻¹¹ to 10⁻³ M. Structure of compounds studied shows Fig. 1.

In the next stage of the experiment, the inotropic and chronotropic responses to imidazolines studied were measured at the presence of fixed concentrations (from 10⁻⁹ to 10⁻³ M) of the imidazoline blockers (idazoxan or/and phentolamine).

Data presentation and statistical evaluation

Cumulative concentration-response curves with variable slope were constructed and analysed by
means of GraphPad Prism4 software (GraphPad Software Inc., San Diego, Ca). Each point of the curves was a mean of at least 6 experiments. The changes of responses to each concentration was expressed as percent of the control value (100 %) of atrial rate or amplitude preceding the administration of cumulative concentrations of an agent with or without idazoxan or phentolamine. Data are reported as mean SEM (standard error of mean). Based on the profile of concentration-response behavior of isolated organs for the compounds studied, the -log EC50 parameters were calculated (the concentration of the ligand producing the half of the maximal effect observed). A significance level was taken as \( p \leq 0.05 \) or \( p \leq 0.01 \) in comparison of the compound studied alone and the same compound pretreated with idazoxan or phentolamine. Nonparametric analysis was done by U’Mann-Whitney (unpaired) test using Statistica 7.1 software (StatSoft Inc., Tulsa, OK, USA).

Data obtained are shown in Figs. 2-6 and in Tables 1-3.

**Drugs**

Pure chemical substances were used for the preparation of bath solutions of the drugs studied.

Phentolamine and idazoxan were obtained from Sigma (Steinheim, Germany); clonidine from Boehringer (Ingelheim, Germany); agmatine [(4-aminobutyl)guanidine], AGN192403 (2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane) and BU239 (2-(4,5-dihydroimidazol-2-yl)quinoxaline) were from Tocris (Bristol, UK); 2-BFI (2-(2-benzofuranyl)-2-imidazoline) was from Tocris (London, UK); rilmenidine was a gift from Servier (Paris, France) and moxonidine was a gift from Dr. B. I. Armah, (BDF Research Laboratories, Hamburg, Germany). Stock solutions of each agent for in vitro studies were \( 10^{-3} \) M. The concentrations of idazoxan were \( 10^{-3} \), \( 10^{-5} \), \( 10^{-7} \) and \( 10^{-9} \) M and phentolamine \( 10^{-9} \) M. Stock solutions were diluted with water ex tempore before individual experiments.

**RESULTS**

**Inotropic activity**

It has been demonstrated that clonidine, moxonidine and rilmenidine elicit the positive inotropic activity on electrically stimulated left atria with maximum effects of 132.1, 116.2 and 118.3 per cent, respectively (Fig. 2A, Table 1). The -log EC50 values observed for clonidine, moxonidine and rilmenidine were 5.2, 6.2 and 5.1, respectively (Table 2).

**Table 1.** Inotropic and chronotropic effects of the cumulative concentrations of the compounds studied. Maximum effect observed are expressed as % of control (at indicated molar concentration).

<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Amplitude of contractility</th>
<th>Rate of contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonidine</td>
<td>132.1±19.8 (10⁻³)</td>
<td>110.2±5.9 (10⁻⁵)</td>
</tr>
<tr>
<td>Moxonidine</td>
<td>116.2±3.1 (10⁻⁵)</td>
<td>99.1±0.9 (10⁻⁵)</td>
</tr>
<tr>
<td>Rilmenidine</td>
<td>118.3±4.7 (10⁻⁷)</td>
<td>100.9±1.2 (10⁻⁴)</td>
</tr>
<tr>
<td>AGN192403</td>
<td>100.4±0.2 (10⁻⁵)</td>
<td>99.1±0.9 (10⁻⁵)</td>
</tr>
<tr>
<td>2-BFI</td>
<td>76.1±8.8 (10⁻⁵)</td>
<td>93.5±4.6 (10⁻⁵)</td>
</tr>
<tr>
<td>BU239</td>
<td>117.4±6.1 (10⁻⁵)</td>
<td>131.0±12.5 (10⁻⁵)</td>
</tr>
<tr>
<td>Agmatine</td>
<td>142.0±9.3 (10⁻³)</td>
<td>125.7±2.4 (10⁻⁵)</td>
</tr>
</tbody>
</table>
The presence of idazoxan 10^{-5} M and 10^{-3} M diminished positive inotropic effect of clonidine and rilmenidine (Figs. 3A, 3C). The -log EC_{50} values for clonidine and rilmenidine increased after pretreatment with idazoxan 10^{-3} and 10^{-5} M (Table 2). Surprisingly, moxonidine produced positive inotropic effect at the presence of idazoxan 10^{-5} M (Fig. 3B). The antagonism at the presence of idazoxan 10^{-3} M manifested itself at the high concentrations (10^{-5} - 10^{-3} M) of moxonidine. The -log EC_{50} for moxonidine alone was 6.2 and it increased to 7.2 after pretreatment with the 10^{-5} M idazoxan (Table 2).

AGN192403 in cumulative concentrations from 10^{-11} to 10^{-3} M does not act on amplitude of beating of left atria. A pretreatment with idazoxan 10^{-9} and 10^{-6} M or phentolamine 10^{-8} M increases inotropic activity of AGN 192403 but these effects are of no statistical significance.

Positive inotropic effect of compound 2-BFI appeared only at very low agent's concentrations (10^{-11}-10^{-8} M). In higher concentrations, 2-BFI decreased the amplitude of contraction of left atria up to 76.1 %, in comparison to the 100% of control. The presence of idazoxan 10^{-9} M partially diminished inotropic effect of 2-BFI but

Table 2. The -log EC_{50} value for compounds studied alone and at the presence of idazoxan (Ida) or phentolamine (Phen).

<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Amplitude of contractility -log EC_{50}</th>
<th>Rate of contraction -log EC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonidine (Clo)</td>
<td>5.2</td>
<td>10.6</td>
</tr>
<tr>
<td>Clo +Ida 10^{-5}</td>
<td>5.7</td>
<td>8.9</td>
</tr>
<tr>
<td>Clo +Ida 10^{-3}</td>
<td>9.8</td>
<td>8.9</td>
</tr>
<tr>
<td>Moxonidine (Mox)</td>
<td>6.2</td>
<td>n.a</td>
</tr>
<tr>
<td>Mox +Ida 10^{-5}</td>
<td>7.2</td>
<td>n.a</td>
</tr>
<tr>
<td>Mox +Ida 10^{-3}</td>
<td>5.3</td>
<td>n.a</td>
</tr>
<tr>
<td>Rilmenidine (Ril)</td>
<td>5.1</td>
<td>n.a</td>
</tr>
<tr>
<td>Ril +Ida 10^{-5}</td>
<td>7.2</td>
<td>n.a</td>
</tr>
<tr>
<td>Ril +Ida 10^{-3}</td>
<td>9.9</td>
<td>n.a</td>
</tr>
<tr>
<td>AGN192403 (AGN)</td>
<td>7.5</td>
<td>7.0</td>
</tr>
<tr>
<td>AGN +Ida 10^{-9}</td>
<td>10.6</td>
<td>7.5</td>
</tr>
<tr>
<td>AGN +Ida 10^{-6}</td>
<td>n.a</td>
<td>3.9</td>
</tr>
<tr>
<td>AGN + Phen 10^{-9}</td>
<td>n.a</td>
<td>4.0</td>
</tr>
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</table>
concentration 10^{-5} M of idazoxan remained without effect on 2-BFI (Fig. 4A). The -log EC_{50} values of 2-BFI alone and pretreated with idazoxan 10^{-5} M were equally 7.0, and for 2-BFI pretreated with idazoxan 10^{-3} M both values were 9.5 (Table 3).

Compound BU239, which is structurally related to 2-BFI and has been reported to label I_{2} receptors, produced positive inotropic activity with the maximum effect observed of 117.4\% (Fig. 4B). Idazoxan at concentration 10^{-7} M slightly diminished the left atria amplitude of contraction evoked by BU239 whereas idazoxan 10^{-9} M increased the activity of BU239. The presence of phentolamine 10^{-9} M had no effect on inotropy of BU239. The -log EC_{50} values were also diminished from 8.8 for BU239 alone to 8.3 for BU239 with idazoxan 10^{-5} M, to 6.6 for BU239 with idazoxan 10^{-3} M and to 7.2 for BU239 with phentolamine 10^{-3} M (Table 3).

The most marked positive inotropy was observed for agmatine with maximal effect observed of 142.0\% and the -log EC_{50} of 8.2 (Fig. 4C, Table 3). Pretreatment with idazoxan 10^{-7} M or 10^{-3} M agmatine decreased the amplitude of left atria. Phentolamine 10^{-9} M antagonized the inotropism of

<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Amplitude of contractility -log EC_{50}</th>
<th>Rate of contraction -log EC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-BFI</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>2-BFI +Ida 10^{-5}</td>
<td>7.0</td>
<td>8.1</td>
</tr>
<tr>
<td>2-BFI +Ida 10^{-3}</td>
<td>9.5</td>
<td>7.1</td>
</tr>
<tr>
<td>BU239 (BU)</td>
<td>8.8</td>
<td>7.3</td>
</tr>
<tr>
<td>BU +Ida 10^{-9}</td>
<td>8.3</td>
<td>7.0</td>
</tr>
<tr>
<td>BU +Ida 10^{-7}</td>
<td>6.6</td>
<td>8.3</td>
</tr>
<tr>
<td>BU +Ida 10^{-5}</td>
<td>n.a.</td>
<td>7.7</td>
</tr>
<tr>
<td>BU + Phen 10^{-9}</td>
<td>7.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Agmatine (Agm)</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Agm +Ida 10^{-9}</td>
<td>7.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Agm +Ida 10^{-7}</td>
<td>7.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Agm +Ida 10^{-5}</td>
<td>n.a.</td>
<td>5.4</td>
</tr>
<tr>
<td>Agm +Ida 10^{-3}</td>
<td>7.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Agm + Phen 10^{-9}</td>
<td>6.7</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Table 3. The -log EC_{50} value for compounds studied alone and at the presence of idazoxan (Ida) or phentolamine (Phen).
Agmatine also. The -log EC_{50} values of agmatine with antagonists were diminished as has been presented in Table 3.

Idazoxan in cumulative concentrations did not affect the amplitude of beating of left atria: the maximum effect was 105.7%. Also phentolamine remained without effect on the inotropic effect of left atria.

**Chronotropic activity**

Clonidine produced a weak positive chronotropic effect on the right atria up to maximum of 110.2% (Figs. 2B and 5A). Idazoxan at concentrations 10^{-4} and 10^{-3} M markedly antagonized positive chronotropic activity of clonidine and decreased the -log EC_{50} value about 100-fold (Table 2).
Rilmenidine and moxonidine had no effect, either alone or pretreated with idazoxan, on right atria (Figs. 5B, 5C).

AGN192403 increased weakly the rate of beating of the right atria (maximum effect was 110.4 %). In experiments with preexposure to idazoxan $10^{-9}$ M or $10^{-6}$ M, the antagonism with regards to chronotropic action occurred with the statistical significance of $p<0.05$. In the case of preexposure with phentolamine $10^{-9}$ M, there was no change of this effects (Fig. 5D). The -$\log EC_{50}$ values were changed for AGN192403 when pretreated with idazoxan $10^{-6}$ M and with phentolamine $10^{-9}$ M, from 7.0 to 3.9, and 4.0, respectively.

2-BFI decreased weakly the rate of beating of the right atria (from maximum effect of 106.5 to minimum of 93.0 %) (Fig. 6A). In the presence of various concentrations of idazoxan, the chronotropic effect of 2-BFI was not changed (Fig. 6A). The -$\log EC_{50}$ values from experiments with 2-BFI untreated and treated with either idazoxan or phentolamine were equal in the range from 6.9 to 8.1 (Table 3).

BU239 in cumulated concentrations significantly increased the rate of beating of the right atria and the maximum effect observed was 131.0 %. However, in the presence of idazoxan $10^{-7}$ and $10^{-9}$ M the chronotropic effect of BU239 was reduced (Fig. 6B). Phentolamine $10^{-9}$ M, added to BU239 preincubated with idazoxan $10^{-9}$, $10^{-7}$ or $10^{-5}$ M, had no effect. All the values of -$\log EC_{50}$ from experiments involving BU239 were similar, ranging only from 7.3 to 8.3 (Table 3).

Agmatine in cumulative concentrations accelerated the rate of the spontaneously beating right heart atria to a maximum of 125.7 % (Fig. 6C). Idazoxan in concentrations $10^{-3}$, $10^{-5}$ or $10^{-3}$ M attenuated agmatine's positive chronotropic effect. Phentolamine $10^{-9}$ M, added to agmatine pretreated with idazoxan, significantly decreased the rate of beating of the atria. The -$\log EC_{50}$ value decreased about 100-fold (Table 3).

Neither idazoxan nor phentolamine in cumulative concentrations had any marked effect on chronotropic activity of right atria.

**DISCUSSION**

**Inotropy**

In this work manifestations of positive inotropic activity were shown in case of agmatine, clonidine, rilmenidine and moxonidine. The narrow range of -$\log EC_{50}$ values for clonidine, rilmenidine and moxonidine from 5.1 to 6.2 indicates a similar receptor-mediated effect of those drugs. Idazoxan diminished significantly statistically the positive inotropic effect of clonidine and rilmenidine, but not that of moxonidine. The positive inotropic effect of rilmenidine was diminished by idazoxan, unless idazoxan was applied at a high concentration $10^{-3}$M, suggesting that rilmenidine exhibits some selectivity for imidazoline receptors. Rilmenidine is usually considered to be an I$_1$ receptor ligand. However, recent evidences show that it may also label an I$_2$-like site (52). Moreover, it was reported that the hypotensive effect of rilmenidine in humans was potently antagonized by idazoxan, whereas it was weakly or not at all antagonized by yohimbine. At the oral dose of 2 mg, rilmenidine has no effect on beating rate of the heart (53, 54).

Molderings et al. (52) observed that rilmenidine and oxymetazoline are potent full agonists to $\alpha_2$ adrenoeptors in rabbit hearts, whereas in the human atrial appendages both agents are antagonists at the $\alpha_2$ autoreceptors, like rauwolscine and idazoxan are. Prazosin is ineffective in that preparation. The antagonistic activity of rilmenidine towards human $\alpha_{2A}$ adrenoeptors indicates that, in contrast to the suggestion based on rabbit data, the hypotensive effect on humans is not due to activation of $\alpha_{2A}$ adrenoeptors but other, presumably I$_1$ imidazoline, receptors are involved (52). In our work, the increasing -$\log EC_{50}$ values for clonidine and rilmenidine at the presence of idazoxan $10^{-3}$ M, suggest dual interaction of the imidazolines with the $\alpha_2$ adenergic and the imidazoline I$_1$ receptors. In the case of clonidine it would confirm the hypothesis that its positive inotropic effect at low doses is mainly due to a stimulation of postsynaptic $\alpha$ adrenoeptors, whereas additional stimulation of the imidazoline receptors occurs at higher drug doses. Certainly our results do not exclude the possibility that rilmenidine and clonidine elicit their effects through putative presynaptic imidazoline receptors, at least as regards the inotropic activity.

In our study moxonidine elicited a weak positive inotropic effect on left atrium. This finding is in accordance with the results obtained by Raasch et al. (55). Evidently the presence of idazoxan potentiates the inotropic effect of moxonidine. It suggests that moxonidine acts as an agonist-antagonist on both adrenoeptors and imidazoline receptors. The -log $EC_{50}$ value increases in the presence of idazoxan at concentration $10^{-3}$ M, but it decreases when $10^{-3}$ M idazoxan is added to the incubation medium. On the other hand, it has been reported, that moxonidine reduced norepinephrine release independently of I$_1$ receptor, thus suggesting the prominent effect of $\alpha_2$ adrenoeptors in cardiac tissue (52). Moxonidine binds with different affinities to cardiac imidazoline I$_1$ receptors, $\alpha_2$ adrenoeptors (56), and, at some conditions, to $\alpha_1$ adrenoeptors. Raasch et al. (55) explain the increase of contractility of left rat atria by
stimulation of postsynaptic $\alpha_1$ adrenoceptors rather than the imidazoline I$_1$ receptors. However, in experiments consisting of chronic moxonidine treatment of the spontaneously hypertensive rats Mukaddam-Daher and Gutkowska (16) and El-Ayoubi et al. (17) observed the specific binding with moxonidine at the atrial I$_1$ subtype receptors.

A structurally related to moxonidine compound AGN192403 did not change the amplitude of beating of the left atria. However, in the presence of various concentrations of idazoxan, AGN192403 showed a weak nonsignificant positive inotropic effect, potentiated with 10$^{-9}$ M of either phentolamine or idazoxan, similarly as in experiments with moxonidine. Most authors suggest that AGN192403 has no effect on circulatory system. However, its general behaviour may suggest it to be a selective ligand of I$_1$ receptor (15, 57, 58) in the experiments on rat hearts, demonstrated that AGN192403 had no influence on norepinephrine level. According to these authors AGN192403 seems to be an antagonist to the imidazoline I$_1$ receptor and the potentiation of inotropic activity by idazoxan seems to result from synergistic interactions.

In the case of agmatine most important are increases of the amplitude of the left atria contraction. Idazoxan at concentration of 10$^{-7}$, 10$^{-5}$ and 10$^{-3}$ M, and phentolamine at concentration of 10$^{-9}$ M, diminished inotropic activity in a dose dependent manner. Except of imidazoline receptors, agmatine has affinity to both the $\alpha/\beta$ adrenergic and dopaminergic receptors. Its effect is mediated probably by all those receptor sites in cardiac tissue.

Compound 2-BFI and a more potent ligand at I$_2$ imidazoline receptor, BU239, evoked a very weak positive inotropic activity. The presence of idazoxan diminished inotropic effect of 2-BFI and BU239 without statistical significance. The -log EC$_{50}$ values for BU239, 2-BFI and idazoxan were 8.8, 7.0 and 7.0, respectively. The reported binding affinities, K$_i$ for I$_2$ receptors in rat brain membrane are 4.2 and 7 nM for BU239 and 2-BFI, respectively (19). Pharmacometric analysis of the data obtained involving 2-BFI and BU239 lead to conclusion that these ligands cannot be clearly identified as either agonists or antagonists of the I$_2$ receptor. This hypothesis is in accordance with the conclusion from the study on the relaxation of rat jejunum evoked by 2-BFI and idazoxan (59).

In our conclusion, the positive inotropic action on isolated rat heart left atria is with the following rank order for the agents studied: agmatine >> clonidine > BU239 ≥ rilmenidine ≥ moxonidine. Rilmenidine and moxonidine act as partial agonists of the imidazoline I$_1$ receptors. In inotropic effects of these imidazolines both the I$_1$ and $\alpha_2$ receptors are engaged. Inotropic effect of clonidine and agmatine is mostly due to the $\alpha$ adrenoceptors activation. The role of I$_2$ imidazoline receptors is not to convince.

**Chronotropy**

Agmatine and clonidine were found to elicit positive chronotropic effect on the right rat heart atria. Idazoxan markedly antagonized activity of clonidine, but independently of the dose used. It is well known that clonidine is an agonist not only of the $\alpha_2$ but also of the $\alpha_1$ adrenoceptors, present in the right atria.

Phentolamine 10$^{-9}$ M, added to agmatine, significantly decreased positive chronotropic effect of agmatine. In opposite, in the experiments of some authors (41, 60) agmatine did not influence contractions of isolated rat heart atria. Agmatine has affinity to both the $\alpha$ adrenergic and the imidazoline receptors. Some investigators demonstrated that agmatine recognizes $\alpha_2$ adrenoceptors but is without effect on these receptors (61). To date, there are no proofs of action of agmatine attributable to agonism or antagonism at the site in vitro.

In the present study, rilmenidine and moxonidine had almost no effect on the spontaneously beating right atria. Pretreatment with idazoxan attenuated the chronotropic effect of the drugs, but this antagonism against the chronotropic effect of rilmenidine and moxonidine never reached statistical significance, presumably because the induced effects were very small. Moxonidine and rilmenidine are the most selective agonists for I$_1$ receptors among imidazoline agents. Nevertheless, some authors classify moxonidine and rilmenidine among selective $\alpha_2$ adrenoceptor agonists claiming that this receptor may be predominant in the chronotropic activity of the assumed I$_1$ imidazoline receptor agonists.

Compound AGN192403 possesses a small positive chronotropic activity on right atrium and this effect is blocked by idazoxan 10$^{-6}$ M and phentolamine 10$^{-9}$ M as proved by the decreased -log EC$_{50}$ values.

An I$_2$ imidazoline receptor ligand, 2-BFI, had a very weak negative chronotropic activity, probably due to its antagonistic activity towards the I$_2$ receptor subtype (19). After pretreatment with idazoxan the effect of 2-BFI remains unchanged.

Another I$_1$ receptor ligand, BU239, is the most potent of all the agents studied in increasing the beating rate of the right atria. An antagonist, idazoxan, applied at various concentrations diminished this activity in an irregular manner. The -log EC$_{50}$ values determined after the preincubation with idazoxan in different concentrations, were closely similar. It may suggest that the chronotropic mechanism of BU239 involves the imidazoline I$_2$ receptors.
In conclusion, regarding to the maximal effect observed, the positive chronotropic action on isolated rat heart right atria were with the rank order for the agents studied: BU239 ≥ agmatine >> clonidine > AGN192403.

In view of our research, the engagement of imidazoline receptors in the chronotropic response of rat heart atria to imidazoline drugs still remains disputable. Certainly, regarding to chronotropic effect of agmatine and clonidine we feel obliged to acknowledge an involvement of the α2/α1 adrenergic receptors. However, as concerns BU239, the results obtained by us demonstrate that its activity on the rat right heart atrial beating rate is exerted via the imidazoline I2 receptors.

REFERENCES


