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Short communication

In vitro evidence that endothelium-dependent vasodilatation induced by clozapine is mediated by an ATP-sensitive potassium channel

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ABSTRACT

Background: There is a definite association between antipsychotic drugs and arterial hypertension. However, endothelium functions are scarcely considered. This investigation was carried out to study the mechanisms involved in clozapine endothelium-dependent vascular reactivity.
Methods: The experimental animals were male Wistar rats with a mean age of 70–90 days (250–300 g). The endothelium-dependent vascular reactivity was studied by measuring the isometric force and then constructing clozapine concentration-response curves. The force registrations were obtained in the aorta rings with and without the endothelium precontracted with phenylephrine ($PE10^{-6}M$) treatment; this followed incubation for 30 min in "organ chambers" with different inhibitors: L- NAME (nitric oxide/cGMP); indomethacin (PGI2/cAMP); tetraethylammonium (TEA), and specific hyperpolarization blockers (paxillin, apamin, glibenclamide). The data were presented as the mean ± standard error of the mean (SEM) and were compared by one-way ANOVA or two-way ANOVA followed by the Bonferroni *post*-test.
Results: The primary outcomes were: 1) Clozapine-induced endothelium-dependent relaxation was not inhibited by indomethacin, L-NAME, ODQ, and methylene blue (MB); 2) The combination of L-NAME + indomethacin partially prevented the relaxation; 3) Clozapine did not induce relaxation in vessels contracted with KCl; 4) TEA did not block the clozapine-induced relaxation in vessels precontracted with PE (10^{-6} M); 5) The potassium channel blockers paxillin and apamin did not prevent relaxation but glibenclamide did.
Conclusion: Concerning the mechanisms involved in clozapine endothelium-dependent vascular reactivity, the present study suggests that there is synergistic participation that probably occurs through a crosstalk mechanism of the cAMP, cGMPpathways and hyperpolarization.

Introduction

Endothelium-dependent vasodilation in the microvasculature is impaired in patients taking antipsychotics (AP), and some studies have shown an association between endothelium damage with metabolic disorders such as diabetes [1]. In a classic cohort study, Osborn et al. [2] found that in people with severe mental illness (which includes all forms of schizophrenia, bipolar disorder, and delusional disorders), AP drugs were associated with an increased risk for coronary heart disease (CHD) mortality and stroke. In depressed patients, cardiac death due to CHD is twice as high as in non-depressed coronary patients, which ispossibly due to endothelial dysfunction. In fact, in an animal model of moderate chronic stress depression, Bouzinova et al. [3] found a positive association between endothelial dysfunction and depression-like symptoms in a chronic mild stress model of depression.

Although hypotension is a frequent side effect of the antidepressant medication, it is unclear whether it can be attributed to interactions within the central nervous or the cardiovascular system [3]. Patients who receive psychopharmacological treatment for a prolonged time can experience preoperative and postoperative orthostatic hypotension as well as severe intraoperative hypotension refractory to therapy. These issues may be of relevant in anaesthesia procedures because anaesthetic agents alone produce hypotension, and a combination of both agents may synergistically affect blood pressure [4,5]. Some

Abbreviations: cAMP, adenosine 3′,5′cyclic monophosphate; cGMP, guanosine 3′,5′cyclic monophosphate; Indo, indomethacin; KCl, potassium chloride; L-NAME, Nω nitro-L-arginine methyl ester; MB, methylene blue; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, H[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; PGI2, prostacyclin; TEA, tetraethylammonium.

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investigations of typical antipsychotics with low potency for the dopamine D2 receptor, *e.g.*, phenothiazine, chlorpromazine, and thioridazine, indicate these treatments are the most likely to cause orthostatic hypotension [6]. Most investigations focused on the intrinsic antagonistic action on adrenergic receptors to explain the mechanism of hypotension. However, a possible vascular direct effect fromthe production of vasorelaxant factors by the endothelium, mainly NO/cGMP, has yet to be investigated.

Clozapine (CLZ) is the "prototypical" second-generation AP drug, which are also named "atypical antipsychotics". Hence, CLZ was widely viewed as the most significant advance in the treatment of schizophrenia since the discovery of the first AP drugs (chlorpromazine and haloperidol in the 1950s and 1960s, respectively). Thus, this investigation was carried out to observe if the antipsychotic clozapine directly influences vascular tone in an *in vitro* model of an isolated rat aorta.

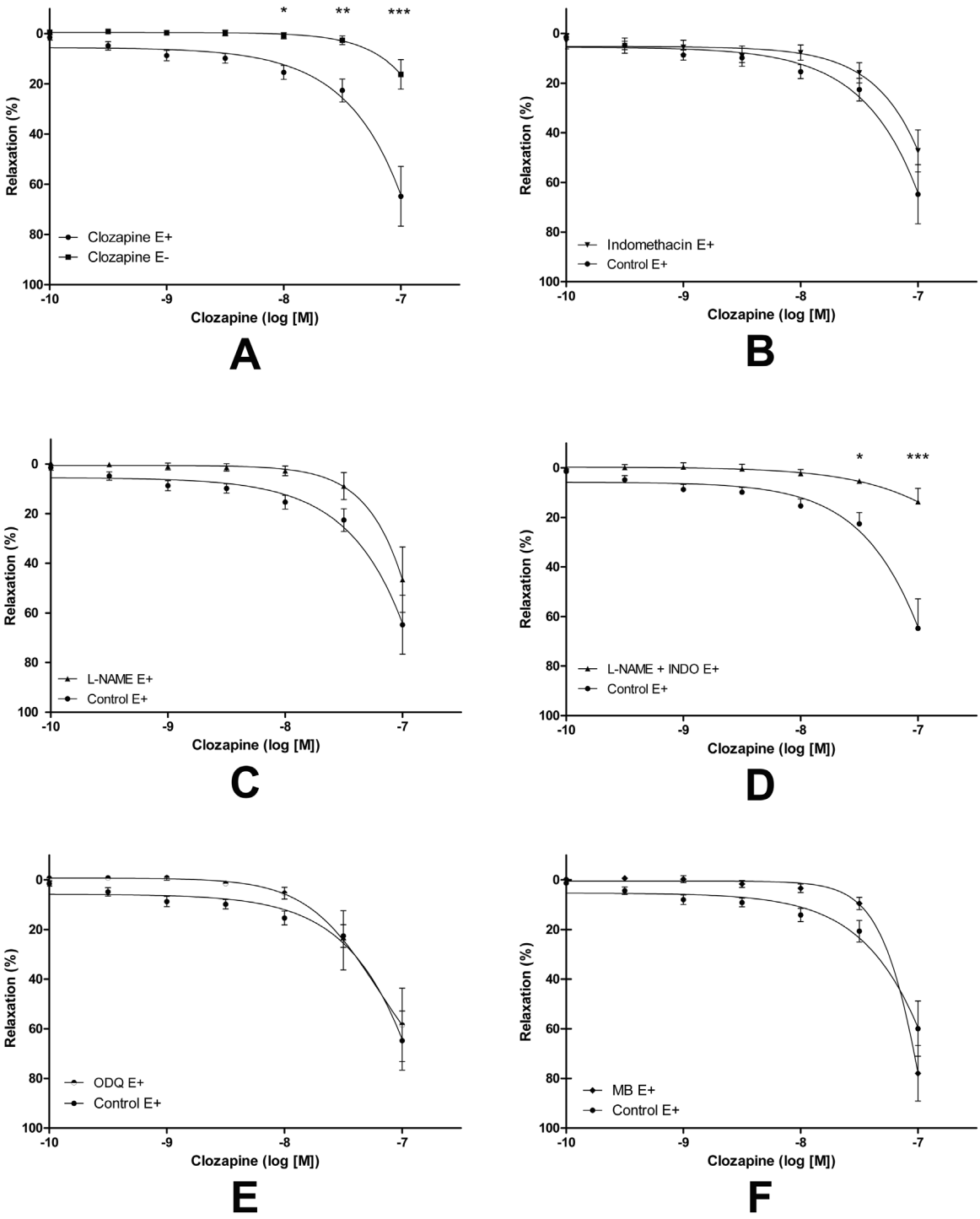

Fig. 1. PGI2/cAMP and NO/cGMP pathway concentration-response curves in rat thoracic aorta precontracted with PE (10^{-6}M). A – Clozapine (10^{-10} to 10^{-7} M) with and without endothelium; B – Indomethacin (10^{-4} M); C - L-NAME (10^{-4} M); D - Indomethacin plus L-NAME (10^{-4} M); E– ODQ (10^{-5} M); F – Methylene blue (10^{-5} M). The compounds were added to the organ bath at least 40 min before contraction with PE. The values represent the mean ± SEM (n = 7). Two-way ANOVA, Bonferroni *post*-test. An asterisk denotes a significant difference from control endothelium-dependent relaxation due to clozapine (* $p<0.05$ ** $p<0.01$ *** $p<0.001$). E+ = with endothelium and E- = without endothelium.

Materials and methods

Animals

The experimental procedures and animal handling were reviewed and approved by the Institutional Committee for Animal Care and Use of the School of Medicine of RibeirãoPreto, University of São Paulo (protocol 208/2016), and the protocols follow the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication n. 85–23, revised 1996). Male Wistar rats with an age of 70–90 days (250–300 g) were housed under standard laboratory conditions (12 h light/dark cycle at 22 °C) with free access to food and water.

Study of vascular reactivity in organ chambers

The rats were anaesthetized with urethane (40 mg/kg, intraperitoneal) and exsanguinated by abdominal puncture of the aorta. Then, the thoracic aorta was carefully removed, placed on a Petri dish filled with Krebs solution, and cut into 4-mm long segments. The rings were placed immediately in an organ chamber filled with 10 ml of Krebs solution with the following composition (listed in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.22, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and $C_6H_{12}O_6$ 11.1, pH 7.4.

The vessels were kept at 37 °C and continuously gassed with a carbogenic mixture (95% O2 and 5% CO2). The tension was measured isometrically with a force transducer (Grass Force-displacement transducer FT03, Grass Instrument CO, Quincy, MA, USA) connected to a data acquisition system (Gould, Cleveland, OH, USA). The rings were gradually stretched to a basal tension of 2.0 g and then allowed to equilibrate for 60 min. In some preparations, the endothelium was mechanically removed by gently rolling the lumen of the vessel on a thin wire.

Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (Ach) 10^{-6} M in the presence of a contractile tone induced by phenylephrine (PE) 10^{-7} M. For studies of endothelium-intact vessel rings, a ring was discarded if its relaxation with Ach did not equal or exceed 80%. Cumulative concentration-response curves were generated in the aorta with and without the endothelium using clozapine (10^{-10} to 10^{-6} M) in an artery pre-contracted with KCl (45 mM) and PE (10^{-7} M).

Experiments were conducted in the presence and absence of Indomethacin (10^{-5} M), which is a non-selective cyclooxygenase (COX) inhibitor, and the NO-synthase inhibitor NG-nitro-L-arginine to evaluate the NO/cGMPmethyl ester (L-NAME, 10^{-4} M), which is a non-selective NOS inhibitor. Additionally, H-[1,2,4] Oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ) (10^{-5} M) and methylene blue (MB) (10^{-5} M), which are sGC inhibitors, were assayed to investigate pathway involvement. To study the participation of K + channels, tetraethylammonium (TEA), apamin (10^{-6} M), paxillin (10^{-6} M) and glibenclamide (10^{-5} M) were used as Small conductance calcium-activated potassium channels (SKCa), Large conductance calcium-activated potassium channels (BKCa), and ATP-sensitive potassium channel (KATP) blockers, respectively. Arterial segments were incubated with each inhibitor for 30 min before constructing a concentration-response curve for clozapine in PE pre-contraction rings.

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM) and were compared by one-way ANOVA or two-way ANOVA, followed by the Bonferroni *post*-test using GraphPad Prism version 4.0 (GraphPad Software Corporation, La Jolla, CA, USA). The adopted level of significance selected was $p < 0.05$.

Results

Clozapine promoted concentration-dependent relaxation in the aorta only in endothelium-intact rings (Fig. 1), and this relaxation was observed when the vessel was pre-contracted with PE but not KCl (Fig. 3). The incubation with indomethacin and L-NAME did not prevent the relaxation induced by clozapine, but the combination of both prevented the relaxation (Fig. 2) partially. Still considering the NO pathway, the ODQ and MB did not block the relaxation by clozapine (Fig. 2).

Concerning hyperpolarization, which was not directly measured, the sensitivity of the relaxation to some channel blockers was investigated. It was observed that only glibenclamide was able to inhibit the clozapine relaxation, but other potassium channels blockers, such as apamin, paxillin or TEA could not (Figs. 3 and 4). The Rmax (%) and pEC50 (log [M]) results are presented on the Table 1.

Discussion

The study was designed to investigate the possible mechanisms of vasoreactivity clozapine-induced in the rat artery aorta *in vitro*. The design considered three known endothelium-dependent pathways (NO/cGMP, PGI2/cAMP, and hyperpolarization). The investigation had the exclusive purpose of observing endothelium-dependent arterial reactivity (rat thoracic aorta) without considering any other cardiovascular function (myocardial contractility, heart rate or autonomic control) [7].

The primary outcomes were: 1) Clozapine-induced endothelium-dependent relaxation was not inhibited by indomethacin, L-NAME, ODQ, and methylene blue (MB); 2) The combination of L-NAME + indomethacin partially prevented the relaxation; 3) Clozapine did not induce relaxation in vessels contracted with KCl; 4) TEA did not block the clozapine-induced relaxation in vessels precontracted with PE (10^{-6}M); 5) The potassium channel blockers paxillin and apamin did not prevent relaxation, but glibenclamide blocked relaxation.

These results together demonstrate that in the rat aorta, endothelium-dependent relaxation elicited by clozapine is not mainly mediated by the NO endothelium-derived relaxation. It was

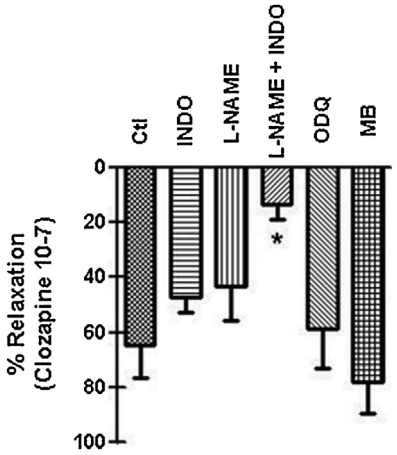

Fig. 2. PGI2/cAMP and NO/cGMP pathways as well as maximal endothelium-dependent relaxation due to clozapine. Rat thoracic aorta segments with endothelium were contracted with PE (10^{-6}M). When indomethacin (10^{-5} M), L-NAME (10^{-4} M), L-NAME (10^{-4} M) plus indomethacin (10^{-4} M), ODQ (10^{-5} M), and Methylene blue (10^{-5} M) were used, the compounds were added to the organ bath at least 40 min before contraction with PE. The values are presented as the means ± SEM. An asterisk denotes a significant difference between the control and L-NAME, plus indomethacin endothelium-dependent relaxation due to clozapine (* $p < 0.05$). E+ = with endothelium.

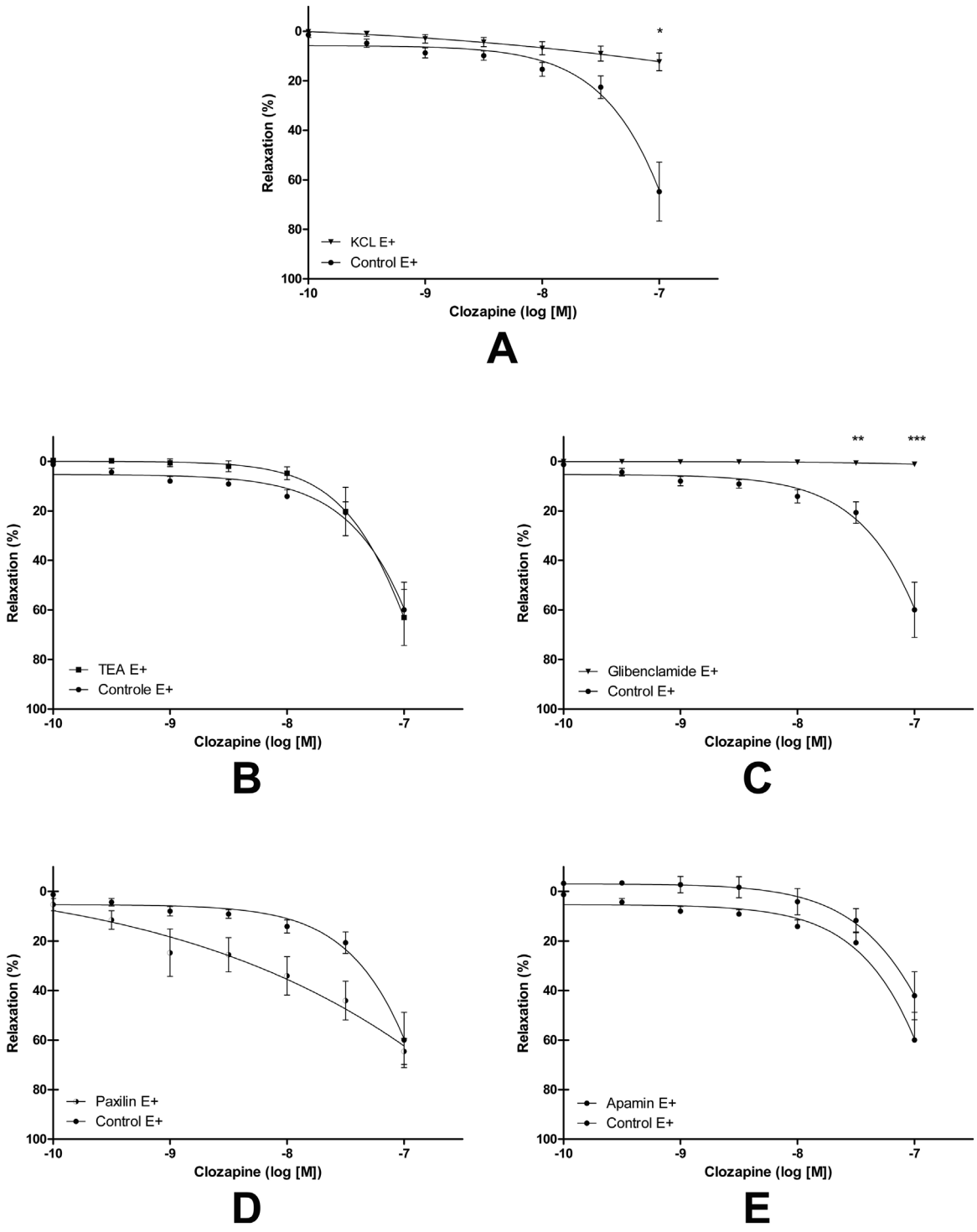

Fig. 3. Hyperpolarization pathway concentration-response curves in rat thoracic aorta. Rat thoracic aorta segments with endothelium were contracted with potassium chloride (KCl 40 mM) or PE (10^{-6}M). A - Endothelium-dependent relaxation to clozapine (10^{-10} to 10^{-7} M); B - tetraethylammonium (TEA) (10^{-3} M); C – Glibenclamide (10^{-5} M); D – Paxillin (10^{-6}M); and E – Apamin (10^{-6} M). The compounds were added to the organ bath at least 40 min before contraction with PE. The values represent the mean ± SEM (n = 7). Only glibenclamide blocked the relaxation with a significant difference ($p < 0.05$). Two-way ANOVA, Bonferroni *post*-test. E+ = with endothelium. An asterisk denotes a significant difference from control endothelium-dependent relaxation due to clozapine (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$); E+ = with endothelium.

necessary to block both the NO pathway (by L-NAME) and the COX pathway (by indomethacin) to reduce the clozapine-induced relaxation. The failure of L-NAME or indomethacin to produce any alteration of the effect of clozapine is not likely to be due to their inability to block NO or prostacyclin (PGI2) release or a similar effect. Both are non-specific-blockers, but the inhibition of the clozapine endothelium-dependent relaxation by the combination of two blockers (L-NAME + indomethacin) suggests that the NO pathway was blocked by both blockers that were used in the experimental conditions. The results indicate that simultaneous inhibition of the production of both NO and COX-derived prostaglandin(s) was required to reveal their involvement. Otherwise, concerning their ability to inhibit receptor-mediated events involving the liberation of NO, analogues of L-arginine

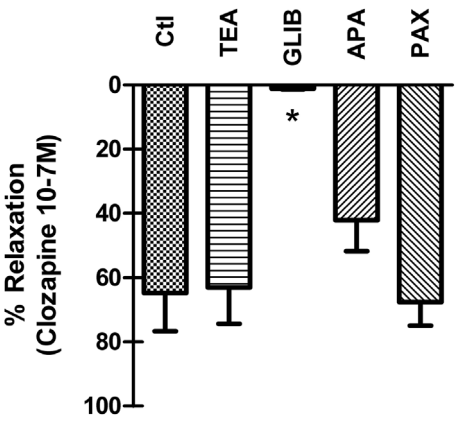

Fig. 4. Hyperpolarization pathways and maximal endothelium-dependent relaxation from clozapine. Endothelium-dependent relaxation from clozapine. Rat thoracic aorta segments with endothelium were contracted with PE (10^{-6}M). When TEA, glibenclamide, paxillin, and apamin were used, the compounds were added to the organ bath at least 40 min before contraction with PE. The values are presented as the means ± SEM. An asterisk denotes a significant difference from control endothelium-dependent relaxation due to clozapine (* $p < 0.05$).

Table 1
Values (mean ± standard deviation) of Rmax (%) and pEC50 (log [M]).

Clozapine	Rmax (%)	pEC50 (log [M])
E+	64.78 ± 11.90	7.20
E-	16.19 ± 5.86	7.10
E + and L-NAME	46.60 ± 13.18	7.15
E + and Indo	47.28 ± 8.47	7.15
E + and L-NAME + Indo	13.74 ± 5.49	7.50
E + and ODQ	58.44 ± 14.79	7.50
E + and MB	77.94 ± 11.22	7.05
E + and TEA	63.06 ± 11.29	7.40
E + and glibenclamide	1.17 ± 0.29	7.50
E + and apamin	42.09 ± 9.71	7.45
E + and paxillin	64.58 ± 5.27	8.00

demonstrate tissue and agonist dependence. This behaviour could reflect agonist efficacy differences in the studied receptor systems. This possibility should be ruled out before apparent resistance to inhibition is taken as evidence for the involvement of different endothelium-derived relaxing factors (EDRFs) [8].

Another possibility would be to consider the interference of clozapine in the crosstalk mechanisms between the cGMP and cAMP systems as well as the main pathways involved in their respective cyclic nucleotide cascades. Large-scale cGMP actions are mediated by activation of the cGMP-dependent protein kinase (PKG), which, conversely, regulates the function of target proteins through phosphorylation. For cAMP, the receptor and the G-protein enzyme associated with adenylatecyclase represent an initial step. Similar tocGMP, the functional effects of cAMP on vascular musculature are mediated primarily by kinase activation. However, not only does it promote cAMP-dependent protein kinase (PKA) activation, but it can also activate PKG. These aspects were not included on the present investigation [9].

Additionally, the capitol role of the classical NO/cGMP pathway was questionable since the sGC inhibitors (ODQ and MB) did not block the clozapine-induced relaxation. However, it is pertinent to emphasize that the absolute real effects of ODQ were questionable since high NO concentrations can overcome the inhibitory effect [10]. Additionally, there is substantial evidence about the low specificity of other inhibitors, such as methylene blue [11–13]

As observed in the present investigation, as well as in many *in vitro* and *in vivo* studies of human and animal arteries, endothelium-dependent relaxation, vasodilatation, and hyperpolarization persist in the presence of L-arginine analogues that are inhibitors of NO synthase (NOS). This outcome has led to the supposition that factors other than NO are important factors in the media [14]. The clozapine endothelium-dependent relaxation also persisted in the presence of TEA. As TEA is a non-specific hyperpolarization blocker, the investigation required testing specific potassium channels blockers: paxillin (voltage and KCa channel inhibitor), apamin (Ca-activated K^+ channel blocker) and glibenclamide (ATP-sensitive potassium channel blocker - KATP) [15–17]. Considering that of these blockers only glibenclamide blocks the clozapine-induced relaxation, there is a possibility that KATP openers alsoact on the smooth muscle as well as the endothelium. This outcome suggests that hyperpolarization of the endothelium may occur simultaneously with smooth muscle relaxation; therefore, it is possible that endothelial hyperpolarization may contribute to the relaxant effects of the K^+ channel activating agents [18]. Therefore, the results of the present *in vitro* investigation do not allow us to rule out the possibility that clozapine acts on some smooth muscle cell target to produce hyperpolarization that would be transmitted to the endothelium. In summary, considering that glibenclamide, but not paxillin or apamin, inhibited the relaxation, it is possible to assume that hyperpolarization mediated by KATP-sensitive potassium channels has a considerable role.

As mentioned previously, the present study was designed only to evaluate possible *in vitro* endothelium-dependent mechanisms of clozapine-induced vasoreactivity, which suggests there should be more studies concerning cardiovascular antipsychotic side effects.

Study limitations

We did not explore the effects of IKCa channel blockers. Without these experiments, the pharmacological profile is incomplete. In the endothelium, increased Ca^{2+} activate NOS and intermediate conductance Ca^{2+} activated K^+ channels (IKCa) results in vasodilation through NOS-mediated NO release or membrane hyperpolarization [19]. Additionally, we did not measure membrane potentials due to the lack of technical resources in our laboratory. The only method used was the force measurement in "organ chambers" with results that led to a simple Short Communication to encourage new investigations.

Conflicts of interest

None.

Acknowledgments

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