β₁- and β₁/β₂-adrenergic receptor antagonists block 6-nitrodopamine-induced contractions of the rat isolated epididymal vas deferens

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Abstract
6-Nitrodopamine (6-ND) is an endogenous modulator of the contractility in the rat isolated epididymal vas deferens (RIEVD) and considered to be the main peripheral mediator of the emission process. Use of selective and unselective β-adrenergic receptor antagonists has been associated with ejaculatory failure. Here, the effects of selective β₁- and β₁/β₂-adrenergic receptor antagonists on RIEVD contractions induced by 6-ND, dopamine, noradrenaline, adrenaline, and electric-field stimulation (EFS) were investigated. The selective β₁-adrenergic receptor antagonists atenolol (0.1 and 1 µM), betaxolol (1 µM), and metoprolol (1 µM) and the unselective β₁/β₂-adrenergic receptor antagonists propranolol (1 and 10 µM) and pindolol (10 µM) caused significant rightward shifts of the concentration–response curve to 6-ND (pA₂ 6.41, 6.91, 6.75, 6.47, and 5.74; for atenolol, betaxolol, metoprolol, propranolol, and pindolol), but had no effect on dopamine-, noradrenaline-, and adrenaline-induced contractions. The effects of selective β₁- and β₁/β₂-adrenergic receptor antagonists at a higher concentration (atenolol 1 µM, betaxolol 1 µM, metoprolol 1 µM, propranolol 10 µM, and pindolol 10 µM) also reduced the EFS-induced RIEVD contractions in control, but not in RIEVD obtained from L-NAME-treated animals. The selective β₁-adrenoceptor agonist RO-363, the selective β₂-adrenoceptor agonist salbutamol, and the selective β₃-adrenoceptor agonist mirabegron, up to 300 µM, had no effect on the RIEVD tone. The results demonstrate that β₁- and β₁/β₂-adrenoceptor receptor antagonists act as 6-ND receptor antagonists in RIEVD, further confirming the main role of 6-ND in the RIEVD contractility.

Keywords Ejaculation disorder · Nitrocatecholamines · EFS · L-NAME

Introduction
6-Nitrodopamine (6-ND) is a novel catecholamine released from vascular tissues such as human umbilical cord vessels (Britto-Jr et al. 2021a), Chelonoidis carbonaria aorta (Campos et al. 2020), and from rat vas deferens (Britto-Júnior et al. 2021b, 2022). The synthesis/release of 6-ND is coupled to nitric oxide (NO) synthesis, since it is reduced by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME).

In the rat epididymal vas deferens, 6-ND has been characterized as a major endogenous modulator of the contractility of this tissue (Britto-Jr et al. 2021b; Britto-Jr et al. 2022). Tricyclic antidepressants such as clomipramine (Millan et al. 2001), desipramine (Cusack et al. 1994), and amitriptyline (Sánchez and Hyttel 1999) and α₁-adrenergic receptor antagonists such as doxazosin (Elliott et al. 1982; Wilt and MacDonald 2006), tamsulosin (Lepor et al. 1988; Dunn et al. 2002), terazosin (Frishman et al. 1988; O’Leary 2001), and alfuzosin (Ramsay et al. 1988) act as 6-ND receptor antagonist in the rat vas deferens (Britto-Jr et al. 2021b; 2022). One known adverse reaction of these two classes of drugs is the impairment of the ejaculatory process.
Beaumont 1977; Cavallini 1995; Hsieh et al. 1999; Debruyne 2000). Indeed, both classes of drugs are used for the treatment of premature ejaculation (Hellstrom and Sikka 2006; Basar et al. 2005), indicating a major role for 6-ND in the ejaculatory process. In pre-clinical studies, male rats treated for 16 weeks with the non-selective β-adrenoceptor antagonist propranolol (1.25 mg/day) exhibited an impairment in the ejaculation and copulatory pattern (Srilatha et al. 1999). Subcutaneous administration of the non-selective β-blocker pindolol (4 mg/kg, 30 min) to male rats was also associated with inhibition of the sexual behavior, as evidenced by an increase in mounts, intromissions, and time to ejaculate (Ahlenius and Larsson 1991). In patients with arterial hypertension, coronary artery disease, or heart failure, meta-analytic data have shown that β-blockers are associated with a small, but significant, increase in risk of reported sexual dysfunction, which was not related to the lipid-soluble β-blockers (Ko et al. 2002). The use of the β1-, β2-, and α1-adrenergic receptor antagonist labetalol was associated with ejaculatory failure soon after the initiation of therapy that resolved with drug discontinuation (O’Meara and White 1988). In a double-blind, placebo-controlled trial comprising eighty-six paroxetine-refractory patients, pindolol, at the dose of 7.5 mg/day, increased significantly the mean intravaginal ejaculatory latency time after 6 weeks of treatment (Safarinejad 2008). Thus, both the experimental and clinical observations open the interesting possibility that β-blockers could act as 6-ND receptor antagonists in the vas deferens, as observed with tricyclic antidepressants and α1-adrenergic receptor antagonists.

Materials and methods

Animals

Adult male Wistar rats (280–320 g) were obtained from the animal care of University of Campinas (UNICAMP; Campinas, São Paulo, Brazil) and Animais de Laboratorio Criação e Com. LTDA (ANILAB; Paulinia, São Paulo, Brazil). All experimental protocols were authorized by the Ethics Committee in Animal Use of UNICAMP (CEUA/UNICAMP, protocol numbers 5952–1/2022 and 5831–1/2021) and followed the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al. 2020). Animals were housed in cages (three per cage) located in ventilated cage shelters with constant humidity of 55% ± 5% and temperature of 24 ± 1 °C under a 12-h light–dark cycle. Animals received filtered water and standard food ad libitum.

Chronic L-NAME treatment

Animals were treated with L-NAME dissolved in the drinking filtered water at a concentration of approximately 20 mg/rat/day for a minimum of 4 weeks (Ribeiro et al. 1992). Control animals received filtered water alone. Vas deferens obtained from these chronically treated animals present lower release of 6-ND, as quantified by LC–MS/MS (Britto-Júnior et al. 2021a, 2021b).

Rat isolated epididymal vas deferens (RIEVD) isolation and preparation

Euthanasia was performed by isoflurane overdose, in which animals were exposed to a concentration greater than 5% until 1 min after the breathing stops. Exsanguination was performed to confirm the euthanasia. The vas deferens was removed and immediately placed in Krebs–Henseleit’s solution (KHS). The proximal portion of the vas deferens (close to the epididymis) was surgically dissected (length, 1.5 cm each) for the functional studies (Burnstock and Verkhratsky 2010). The RIEVD strips were suspended vertically between metal hooks in 10-mL custom designed glass chambers containing KHS, continuously gassed with a mixture of 95%O2:5%CO2 at 37 °C using a heated circulator (PolyScience, IL, USA). Tissues were allowed to equilibrate under a resting tension of 10 mN, and the isometric tension was registered using a PowerLab system (ADInstruments, Sydney, Australia). Following a 45-min stabilization period, the RIEVD strips were initially contracted with a single concentration of noradrenaline (NA, 10 µM) to verify the tissue viability.

In vitro functional assays in RIEVD preparations

Cumulative concentration–response curves to 6-ND were performed in RIEVD strips in the absence and the presence of atenolol (0.1 and 1 µM, 30 min), betaxolol (0.1 and 1 µM, 30 min), metoprolol (0.1 and 1 µM, 30 min), propranolol (1 and 10 µM, 30 min), or pindolol (1, 3 and 10 µM, 30 min). In separate RIEVD preparations, cumulative concentration–response curves to dopamine, noradrenaline, and adrenaline were performed in the absence and presence of atenolol (1 µM, 30 min), betaxolol (1 µM, 30 min), metoprolol (1 µM, 30 min), propranolol (10 µM, 30 min), or pindolol (10 µM, 30 min).

Cumulative concentration–response curves to selective β1-adrenoceptor agonist RO-3630 (0.001–300 µM),
selective β2-adrenoceptor agonist salbutamol (0.001–300 µM), and selective β3-adrenoceptor agonist mirabegron (0.001–300 µM) were performed in RIEVD strips obtained from control animals.

Electric-field stimulation (EFS) in RIEVD preparations

The EFS-induced contractions from RIEVD were evaluated in control and L-NAME-treated rats. Briefly, RIEVD strips were submitted to EFS (60 V for 20 s, at 2–16 Hz in square-wave pulses, 0.3 ms pulse width, and 0.1 ms delay), using a Grass S88 stimulator (Astro-Medical, Industrial Park, RI, USA). In control animals, EFS-induced contractions were evaluated in the absence and the presence of atenolol (0.1 and 1 µM, 30 min), betaxolol (0.1 and 1 µM, 30 min), metoprolol (0.1 and 1 µM, 30 min), propranolol (1 and 10 µM, 30 min), pindolol (1 and 10 µM, 30 min), or tetrodotoxin (1 µM). In L-NAME-treated rats, EFS-induced contractions were evaluated in the absence and in the presence atenolol (1 µM, 30 min), betaxolol (1 µM, 30 min), metoprolol (1 µM, 30 min), propranolol (10 µM, 30 min), or pindolol (10 µM, 30 min).

Drugs and solutions

Atenolol, dopamine, metoprolol, mirabegron, N⁰-nitro-L-arginine methyl ester hydrochloride (L-NAME), salbutamol, and propranolol were obtained from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Adrenaline, betaxolol, noradrenaline, pindolol, and tetrodotoxin were purchased from Cayman Chemical Co (MI, USA). 6-Nitrodopamine was bought from Toronto Research Chemicals Inc (Toronto, Ontario, Canada). RO-363 was provided by MedChem Express (NJ, USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl₂), magnesium sulfate (MgSO₄), sodium bicarbonate (NaHCO₃), potassium phosphate monobasic (KH₂PO₄), and glucose were acquired from Merck KGaA (Darmstadt, Germany). The composition of the KHS was in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, and dextrose 5.6.

Data analysis

Nonlinear regression analysis to determine the pEC₅₀ was carried out using GraphPad Prism (GraphPad Software, version 9.0, San Diego, CA, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{\text{max}}/[1 + (10c/10x)^n] + F$, where E represents the increase in response contractile induced by the agonist; $E_{\text{max}}$ is the effect agonist maximum; c is the logarithm of concentration of the agonist that produces 50% of $E_{\text{max}}$; x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line; and F is the response observed in the absence of added drug. The values of pEC₅₀ data represent standard deviation (SD) of n experiments. Values of $E_{\text{max}}$ were expressed in mN. Each animal provided two epididymal vas deferens (right and left); one strip was used as the control response and the contralateral strip was incubated with an antagonist/inhibitor; n indicates both the number of paired strips (same animal) and the number of rats. Student’s two-tailed unpaired t-test was employed and the differences between groups. In addition, standard ANOVA, followed by the Newman–Keuls post-test, were used when more than two groups were involved. A p value less than 0.05 was considered statistically significant. Since the study has an exploratory character, the p values should be considered descriptive (Motulsky 2014; Michel et al. 2020). For 6-ND, the pA₂ values of the antagonists were calculated from the intercept on the concentration axis and by application of the equation: $pA_2 = \log (\text{antagonist concentration}) - \log (\text{CR-1}) - \log (\text{antagonist concentration})$ (Arunlakshana and Schild 1959).

Results

Effect of atenolol on RIEVD contractions induced by catecholamines and EFS

Atenolol (0.1 and 1 µM) produced concentration-dependent rightward shifts on the concentration–response curves to 6-ND (Fig. 1A; $p = 0.0284$ and $p = 0.0068$, respectively) with a pA₂ value of $6.51 \pm 0.54$ (n = 4). Atenolol (0.1 µM) had no effect on the RIEVD contractions induced by dopamine (Fig. 1B; $p = 0.4540$), noradrenaline (Fig. 1C; $p = 0.4234$), and adrenaline (Fig. 1D; $p = 0.4570$).

Atenolol (0.1 µM) had no effect on the EFS-induced contractions of RIEVD (Fig. 1F), but at a higher concentration (1 µM), atenolol caused significant reductions on the EFS-induced contractions of the RIEVD in all frequencies tested (Fig. 1F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 1G).

Effect of betaxolol on RIEVD contractions induced by catecholamines and EFS

Betaxolol at 0.1 µM had no effect on 6-ND-induced RIEVD contractions, but at 1 µM it caused a significant rightward shift on the concentration–response curve to 6-ND (Fig. 2A; $p = 0.0046$) with a pA₂ value of $6.91 \pm 0.03$ (n = 4). Betaxolol (1 µM) had no effect on the RIEVD contractions induced by dopamine (Fig. 2B; $p = 0.4608$), noradrenaline (Fig. 2C; $p = 0.2830$), and adrenaline (Fig. 2D; $p = 0.1571$).
Betaxolol (0.1 µM) had no significant effect on EFS-induced RIEVD contractions (Fig. 2F); however, at 1 µM betaxolol caused significant reductions of the EFS-induced contractions in all frequencies tested (Fig. 2F). In RIEVD obtained from animals chronically treated with L-NAME, betaxolol (1 µM) had no effect on the EFS-induced contractions (Fig. 2G).

Effect of metoprolol on RIEVD contractions induced by catecholamines and EFS

Metoprolol at 0.1 µM had no effect on 6-ND-induced RIEVD contractions, but at 1 µM it caused a significant rightward shift on the concentration–response curve to 6-ND (Fig. 3A; \( p = 0.0159 \)) with a \( pA_2 \) value of 6.75 ± 0.08 (n=4). Metoprolol
Effect of betaxolol on RIEVD contractions induced by catecholamines and EFS

Betaxolol (1 µM) caused significant concentration-dependent rightward shifts of the concentration–response curves to 6-ND (A). Betaxolol (1 µM) had no effect on the RIEVD contractions induced by dopamine (DA; B), noradrenaline (NA; C), and adrenaline (ADR; D) concentration–response curves. Metoprolol (0.1 µM) had no effect on the EFS-induced contractions (E), but at higher concentration (1 µM) significantly reduced the contractions in all frequencies tested (F). In RIEVD obtained from animals chronically treated with L-NAME, betaxolol (1 µM) failed to affect the EFS-induced contractions (G). Data are expressed as mean ± SD. *p < 0.05 compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in A whereas the unpaired t-test was applied in B–G. n means the number of vas deferens strips.

(1 µM) had no effect on the RIEVD contractions induced by dopamine (Fig. 3B; p = 0.4540), noradrenaline (Fig. 3C; p = 0.1887), and adrenaline (Fig. 3D; p = 0.3795).

Metoprolol at 0.1 µM had no significant effect on EFS-induced RIEVD contractions (Fig. 3E); however, at 1 µM metoprolol caused significant reductions of the EFS-induced contractions in all frequencies tested (Fig. 3F). In RIEVD obtained from animals chronically treated with L-NAME, metoprolol (1 µM) had no effect on the EFS-induced contractions (Fig. 3G).

Effect of propranolol on RIEVD contractions induced by catecholamines and EFS

Propranolol (1 and 10 µM) produced concentration-dependent rightward shifts on the concentration–response
curves to 6-ND (Fig. 4A; \( p = 0.029 \) and \( p = 0.0345 \) for 1 and 10 µM, respectively) with a \( pA_2 \) value of 6.47 ± 0.35 (n = 4). Propranolol (10 µM) had no effect on the RIEVD contractions induced by dopamine (DA; B), noradrenaline (NA; C), and adrenaline (ADR; D) concentration–response curves. Metoprolol (0.1 µM) had no effect on the EFS-induced contractions (E), but at higher concentration (1 µM) significantly reduced the contractions in all frequencies tested (F). In RIEVD obtained from animals chronically treated with L-NAME, metoprolol (1 µM) failed to affect the EFS-induced contractions (G). Data are expressed as mean ± SD. *\( p < 0.05 \) compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in A whereas the unpaired t-test was applied in B–G, n means the number of vas deferens strips.

Propranolol at 1 µM had no significant effect on EFS-induced RIEVD contractions (Fig. 4E); however, at 10 µM propranolol caused significant reductions of the EFS-induced contractions at the frequencies of 4 to 16 Hz (Fig. 4F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 4G).
Effect of pindolol on RIEVD contractions induced by catecholamines and EFS

Pindolol (10 µM) caused a rightward shift on the concentration–response curve to 6-ND (Fig. 5A; \( p = 0.0184 \)) with a \( pA_2 \) value of 5.74 ± 0.15 (\( n = 4 \)). Lower concentrations of pindolol (1 and 3 µM) had no significant effect on the contractions induced by 6-ND (Fig. 5A). Pindolol (10 µM) had no effect on the RIEVD contractions induced by dopamine (DA; B), noradrenaline (NA; C), and adrenaline (ADR; D) concentration–response curves. Propranolol (1 µM) had no effect on the EFS-induced contractions (E), but at higher concentration (10 µM) significantly reduced the contractions in all frequencies tested (F). In RIEVD obtained from animals chronically treated with L-NAME, propranolol (10 µM) failed to affect the EFS-induced contractions (G). Data are expressed as mean ± SD. *\( p < 0.05 \) compared with respective control values. ANOVA followed by the Newman–Keuls post-test was applied in A whereas the unpaired \( t \)-test was applied in B–G. \( n \) means the number of vas deferens strips.
Pindolol at 1 µM had no significant effect on EFS-induced RIEVD contractions (Fig. 5E); however, at 10 µM, pindolol caused significant reductions of the EFS-induced contractions at the frequencies of 4 to 16 Hz (Fig. 5F), which was not observed in RIEVD obtained from animals chronically treated with L-NAME (Fig. 5G).

**Effect of RO-363, salbutamol, and mirabegron on RIEVD tone**

The selective β₁-adrenoceptor agonist RO-363 (Fig. 6A), the selective β₂-adrenoceptor agonist salbutamol (Fig. 6B), and the selective β₃-adrenoceptor agonist mirabegron (Fig. 6C), up to 300 µM, had no effect on the RIEVD tone.
Effect of tetrodotoxin on RIEVD contractions induced by EFS

Pre-treatment (30 min) with tetrodotoxin (TTX; 1 µM) abolished the EFS-induced contractions in the RIEVD (Fig. 7).

Discussion

The results clearly indicate that both selective and non-selective β-blockers can antagonize the contractions of the rat epididymal vas deferens induced by 6-ND, as observed with α₁-adrenergic receptor antagonists and tricyclic depressants. These findings also reinforce the role of 6-ND as the major modulator of rat epididymal vas deferens contractility, since the contractions induced by electric-field stimulation were inhibited by the β-blockers only at the concentrations that caused right-shifts of the 6-ND concentration–response curves. The inhibition of RIEVD contractions by the β-blockers was not observed in the vas deferens obtained from animals chronically treated with L-NAME, further supporting the concept that the inhibition of EFS-induced by β-receptor antagonists is due to blockade of 6-ND action.

β₁-Adrenergic receptors are not considered relevant for contractile activity in the rat vas deferens, since this tissue contains a homogenous population of β₂-adrenoceptors that inhibit field-stimulated contractions (Vohra 1979). Radioligand binding using [125I]-pindolol in the rat vas deferens labeled a single class of high affinity binding sites with properties consistent with a population of β₂-adrenoceptors (May et al. 1985). In the rat vas deferens, β₂-adrenergic antagonists such as carazolol is more potent to displace [³H]-dihydroalprenolol binding compared to β₁-adrenergic antagonists such as atenolol and practolol (Chang and Lotti 1983). Indeed, the finding that the selective β₁-adrenergic receptor agonist RO-363 (Iakovidis et al. 1980) had no contractile activity per se confirms the relative unimportance of this subclass of receptors in the vas deferens. The lack of contractile activity of RO-363 clearly demonstrates that the contractions induced by 6-ND are not due to activation of β₁-adrenergic receptors. Similar results were obtained with the selective β₂-adrenergic

Fig. 6 Effect of the selective β₁-adrenergic receptor agonist RO-363 (A), selective β₂-adrenergic agonist salbutamol (B), and of the selective β₃-adrenergic agonist mirabegron (C) in the rat isolated epididymal vas deferens tone. n means the number of vas deferens strips.

Fig. 7 Electric-field stimulation (EFS) caused frequency-dependent contractions of the isolated rat epididymal vas deferens (RIEVD), which were abolished by pre-treatment with tetrodotoxin (TTX, 1 µM). Data are expressed as mean ± SD. *p<0.05 compared with respective control values in the unpaired t-test. n means the number of vas deferens strips.
agonist salbutamol and the selective β3-adrenergic agonist mirabegron, indicating that the contractile activity induced by 6-ND is independent of β-adrenergic receptor activation. 6-ND has been considered the endogenous mediator of EFS-induced contractions in the rat vas deferens (Britto-Júnior et al. 2021b), and these results further support the concept that 6-ND is acting on a specific 6-ND receptor.

In the rat vas deferens, the adrenergic axons are clearly identified within smooth muscle cells, and some are completely ensheathed in the smooth muscle cells (Furness and Iwayama 1971). Thus, the high concentrations of β-blockers required to inhibit EFS-induced contractions can reflect restricted access to the β-adrenergic receptors located in deeper layers of smooth muscle cells. However, this anatomical hypothesis is unlikely since EFS-induced contractions are inhibited by much lower concentrations of α-adrenergic antagonists (Britto-Júnior et al. 2022).

Although ejaculatory disorders have been reported with the use of selective and unselective β-blockers, the incidence is rather low (reported cases) when compared to α1-adrenergic receptor antagonists (4–11%; Höfner et al. 1999). This major difference in incidence (Djavan et al. 2004) could be easily attributed to the observed major potency (over 100 times) of α1-blockers (the pA2 values are 9.66, 9.15, 8.86, 7.70, 7.20, and 8.82 for tamsulosin, doxazosin, alfuzosin, silodosin, terazosin, and prazosin; Britto-Júnior et al. 2022) in blocking 6-ND contractile activity compared to the α1-adrenergic antagonists (2021/13593–6).

The inhibitory effect of β1- and β1/β2-adrenergic receptor antagonists on the RIEVD contractions induced by both the EFS and 6-ND is due to blockade of the 6-ND receptor.

Author contribution Conceptualization: JBJ, GDN.

Data curation: JBJ, GDN.

Formal analysis: GDN.

Funding acquisition: EA, GDN.

Investigation: ATL, ACA, JBJ, RRC, GDN.

Methodology: ATL, ACA, JBJ, RRC, AF, EA, FZM, GDN.

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Visualization: AF, EA, GDN.

Writing—original draft: JBJ, AF, EA, GDN.

The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The authors authorize the availability of any data used in this study.

Declarations

Ethics approval All experimental protocols were authorized by the Ethics Committee in Animal Use of UNICAMP (CEUA/UNICAMP, protocol numbers 5952–1/2022 and 5831–1/2021).

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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