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Endogenous Norepinephrine Desensitizes α_{1D}-Adrenoceptors in Cultured Rat Aorta

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Abstract

Desensitization is a process characterized by the loss of cellular response to an agonist when this is present for a long time. α_{1D}-adrenergic receptor (α_{1D}-AR) desensitization is important since this receptor is involved in the contraction of large caliber arteries, such as the aorta. The aim of this research was to evaluate the desensitization of α_{1D}-AR due to the endogenous release of norepinephrine in cultured rat aorta. Wistar rat aorta was incubated for 2 h or 24 h in DMEM at 37˚C, and then subjected to isometric tension and the action of added norepinephrine, in concentration-response curve (CRC). In some experiments, BMY-7378 (α_{1D}-AR antagonist) or 5-methylurapidil (α_{1A}-AR antagonist) was used to identify the α_1-AR involved in the response, or BMY-7378 to protect the α_{1D}-AR from desensitization. Results showed that α_{1D}-AR was desensitized when the aorta was incubated for 24 h, since the CRC to exogenous norepinephrine showed lower maximal contraction and the curve was displaced to the right, indicating that the receptor involved in contraction was not the α_{1D}-AR, as compared to the aorta incubated 2 h. The receptor stimulated by norepinephrine at 24 h was neither the α_{1A}-AR, as shown by the lack of displacement of the curve by 5-methylurapidil, but rather it seems that α_{1B}-AR is inducing contraction. When the aorta was incubated with BMY-7378 for 24 h, the α_{1D}-AR antagonist protected the receptor from desensitization. Endogenous norepinephrine desensitizes α_{1D}-AR in the cultured aorta, and the α_{1D}-AR is protected by BMY-7378.

Keywords

Desensitization, Norepinephrine, α_{1D}-Adrenergic Receptor, BMY-7378, Rat Aorta

1. Introduction

Receptor desensitization is a phenomenon defined as the reduction in a cell's response to persistent stimulation by either an endogenous or exogenous agonist [1] [2] [3]. Receptor desensitization is a relevant physiological process that shuts off G protein-coupled receptors (GPCRs) from overstimulation, in case of prolonged agonist occupancy, attenuating or terminating its signaling. Typically, this phenomenon has been evidenced by administering an exogenous agonist to the cell, organ, or organism, and then measuring the attenuated response to the same agent after prolonged exposure [3] [4]. Various receptor systems exhibit receptor desensitization, including ion channels [5] [6] [7], GPCRs [4] [8], and tyrosine kinase receptors [9] [10]. Furthermore, there are two main modes of desensitization: homologous and heterologous. Homologous desensitization is triggered by a high concentration of the agonist targeting a specific receptor, often mediated by GPCR kinases (GRKs) [11]. In contrast, heterologous desensitization manifests as a diminished response to one or multiple agonists/receptors, typically mediated by protein kinases or downstream components in the signaling pathway [11] [12] [13] [14] [15].

With respect to GPCRs, it is known that the central nervous system releases norepinephrine (NE) from the terminals of noradrenergic neurons to target cells, such as other neurons, muscle cells, and various other cell types [3] [16] [17]. Upon release, NE accumulates in the synaptic cleft, stimulating postsynaptic adrenergic receptors (α_1-AR or β-AR), and may either be reuptake by the presynaptic neuron or act on α_2-AR on the presynaptic neuron to inhibit further NE release [16] [18]. This desensitization, also called tachyphylaxis, is commonly observed in intensive care units of hospitals, but it also occurs continuously in our cells.

Numerous studies have delineated the molecular mechanisms involved in adrenergic receptor desensitization. Here, the administration of exogenous adrenergic agonists, such as norepinephrine, phenylephrine, and amidephrine, among others, has been shown to induce a weakened cell or tissue response [2] [3] [4] [19] [20] [21] [22] [23] [24]. For example, Rat-1 fibroblasts expressing α_{1D}-AR show low calcium mobilization when incubated with phorbol myristate acetate, a PKC activator, and then stimulated with NE, resembling α_{1D}-AR blockade/desensitization (heterologous type) [2].

Contrastingly, there is no documented desensitization for the endogenous stored and released neurotransmitter (NE) at the neuromuscular junction, specifically when it acts on postsynaptic vascular smooth muscle α_1-ARs. A key query arises regarding whether released NE could lead to desensitization of the predominant α_1-AR in rat aorta, *i.e.* the α_{1D}-AR [25] [26] [27]. Since aorta is a poorly innervated conductance artery and expresses α_{1D}-AR as the predominant functional receptor that responds with high sensitivity to NE stimulus, it is very important to comprehend how the α_{1D}-AR desensitization in this vessel could prevent sudden changes due to contraction-relaxation. The goal of this study

was to assess the potential desensitization induced by stored norepinephrine on the α_{1D}-AR of rat aorta, in order to seek α_1-AR regulation when the main receptor is downregulated.

2. Materials and Methods

2.1. Animals and Ethical Statement

Male Wistar rats, aged 3 months and weighing between 250 - 300 g, were housed under pathogen-free conditions with maintained parameters (40% - 60% humidity, 22˚C ± 2˚C and a 12 h light/dark cycle), in our animal facilities. They were provided food *ad libitum*. All animal care and experimental procedures complied with the Mexican Regulations of Animal Care and Use (NOM-062-ZOO-1999, SAGARPA, Mexico), and adhered to the Guide for the Care and Use of Laboratory Animals as set forth by the U.S. National Institutes of Health [28]. The Institutional Ethics Committee of FES Iztacala, UNAM approved all procedures under Protocol 1497.

2.2. Procedures

2.2.1. Incubation Conditions

Rats were euthanized, following which the thoracic aorta was carefully dissected and cleared of adjacent adipose tissue. The isolated aorta was positioned within a Petri dish inside a laminar flow hood. Subsequently, it was sectioned into rings measuring 4 - 5 mm in length. To ensure the exclusion of potential contributions from endothelium-derived factors in the mechanical response, the endothelium was removed by gently rubbing the intima using a metal instrument. The effectiveness of endothelium removal was confirmed by the absence of relaxation in response to carbachol (1×10^{-6} M) [29].

The aortic rings were then submerged in 3 ml of Dulbecco's Modified Eagle Medium (DMEM), within a 6-well culture plate. The plates were subsequently placed in a BB 150 CO_2 incubator set at 37˚C (Thermo Scientific, Waltham, MA, USA), maintaining an atmosphere of 95% air and 5% CO_2, for durations of both 2 h and 24 h.

2.2.2. Concentration-Response Curve (CRC)

The arterial rings were placed in 10 ml chambers filled with Krebs solution, composed of (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11.1. The solution was maintained at 37˚C with a pH 7.4 and continuous oxygenation (O_2/CO_2 ratio of 95%/5%). Each arterial ring was secured at its base within the chamber and linked to an isometric FT03E Grass force displacement transducer (Astro-Med, Inc., West Warwick, RI, USA). This transducer was then connected to a MP100A data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA), to capture the isometric tension developed by the aortic rings. Based on preliminary trials, the arterial rings were set to an initial optimal tension of 3 g. This tension was achieved by methodically increasing the initial tension until the optimal value was identified

[25] [26].

2.2.3. α_1-Adrenergic Receptor Agonism

Upon completion of incubation periods (either 2 h or 24 h) in DMEM, the aortic rings were transferred to the incubation chamber. They were exposed to norepinephrine (1×10^{-7} M) in the simultaneous presence of propranolol (1×10^{-7} M) and rauwolscine (1×10^{-7} M), to antagonize β- and α_2-adrenergic receptors, respectively. This environment was refreshed every 30 min for 2 h, termed the stabilization period. Subsequently, a reproducible cumulative CRC to norepinephrine was established, ranging from 1×10^{-9} M to 1×10^{-5} M using half logarithm increments (termed the control curve).

In a separate experimental series, aortic rings were immersed in DMEM for duration of either 2 h or 24 h. This immersion was accompanied by escalating concentrations of norepinephrine ($1 \times 10^{-8.5}$ M to $1 \times 10^{-6.5}$ M, applied in half logarithm increments). Following this treatment, these rings were placed in the incubation chamber and subsequently challenged with norepinephrine, using concentrations ranging from 1×10^{-9} M to 1×10^{-5} M, in half logarithm steps.

2.2.4. α_1-Adrenergic Receptor Antagonism

To assess the tissue response to α_1-adrenergic receptor (α_1-AR) stimulation under varying incubation conditions, aortic rings that had been incubated in DMEM for either 2 h or 24 h were subjected to increasing concentrations of selective antagonist. Specifically, these included the α_{1A}-AR selective antagonist, 5-methylurapidil; the α_{1D}-AR selective antagonist, BMY-7378; and chloroethylclonidine, which serves as a selective but alkylating antagonist for α_{1B}-AR. The purpose of this regimen was to identify the specific α_1-AR involved in the contractile response to norepinephrine.

2.2.5. Materials

All reagents were prepared either in Krebs solution or distilled water. Solutions were freshly prepared for every experiment. Reagents, including (±) Norepinephrine-HCl, (±) Propranolol-HCl, Rauwolscine-HCl, Carbachol chloride, 5-methylurapidil (5-MU, 5-Methyl-6[[3-[4-(2-methoxyphenyl)-1-piperazinyl]-propyl]amino]-1,3-dimethyluracil), Chloroethylclonidine (CEC, 2-[2,6-Dichloro(N-β-chloroethyl-N-methyl)-4-aminomethyl]phenylimino-2-imidazolidine dihydrochloride), and BMY-7378 (BMY, 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride) were sourced from Sigma-Aldrich (St. Louis, MO, USA). DMEM was obtained from Gibco (Life Technologies Co., Grand Island, NY, USA). All other reagents were of analytical grade and were purchased from local suppliers.

2.2.6. Statistical Analysis

Values for pD_2 (-log EC_{50}) were derived using nonlinear regression, while pA_2 values were determined through Schild analysis [30]. Data are expressed as means ± standard error of the mean (SEM) of 8 rats per group. Statistical evalu-

ations were performed using analysis of variance (ANOVA) or Student's *t*-test, with differences statistically significant at $p < 0.05$.

3. Results

To assess the viability of aortic tissue after incubation (2 h and 24 h at 37˚C in DMEM), contractions were induced using high KCl (80 mM) for each time interval. High KCl is known to depolarize the membrane, facilitating Ca^{2+} entry into muscle cells and thus initiating contraction, a process that is receptor-independent [31]. As shown in **Figure 1**, high KCl induced contraction in aortic rings for both incubation periods, suggesting that the incubation conditions did not affect tissue responsiveness. Separate incubation of the aorta for 24 h in Krebs solution at 4˚C gave a pD_2 of 8.7 when activated by NE (data not shown).

In contrast, **Figure 2** displays the concentration-response curve (CRC) for norepinephrine after incubation times of either 2 h or 24 h at 37˚C in DMEM. The aortic response to norepinephrine demonstrated both higher efficacy (3.71 ± 0.26 g *vs.* 2.57 ± 0.26 g, respectively) and potency (pD_2: 8.62 ± 0.11 *vs.* 6.64 ± 0.13, respectively) for tissues incubated for 2 h as compared to those incubated 24 h. Such findings suggest that a 24 h incubation in DMEM leads to α_1-AR desensitization, likely due to endogenous norepinephrine release at the neuromuscular junction, resulting in 30% decrease in maximal contraction and 2 orders of magnitude in potency. Consequently, we explored the impact of adding ed norepinephrine during incubation, followed by CRC construction.

As shown in **Figure 3**, introducing varying norepinephrine concentrations to the incubation medium across both time intervals led to a further reduction in

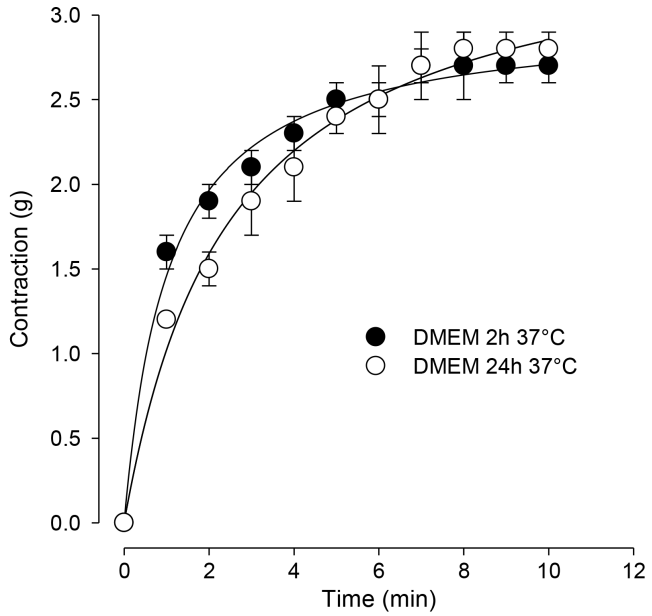

Figure 1. Time-course of aortic contraction induced by high KCl (80 mM) following incubation for 2 h at 37˚C (●) and 24 h at 37˚C (○), both in DMEM. n = 8 rats.

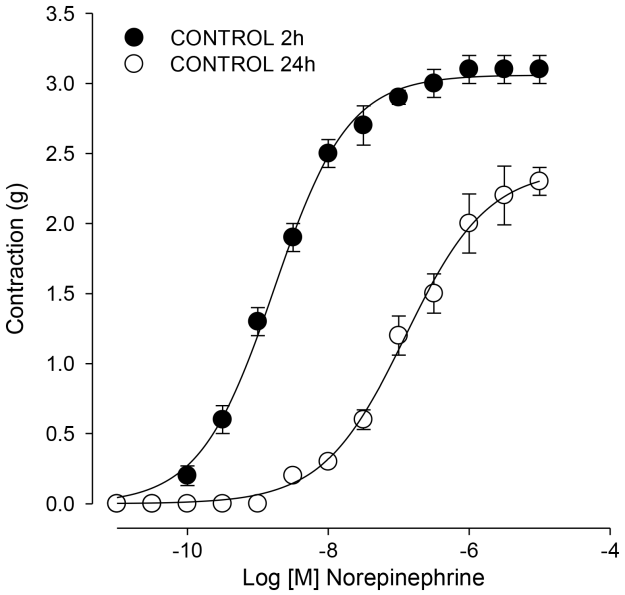

Figure 2. Concentration-response curve to norepinephrine (NE) in the aorta following 2 h and 24 h incubation in DMEM. The curve with (●) represents NE-induced contraction in the aorta after 2 h incubation, while the curve with (○) depicts NE-induced contraction after 24 h incubation. n = 8 rats.

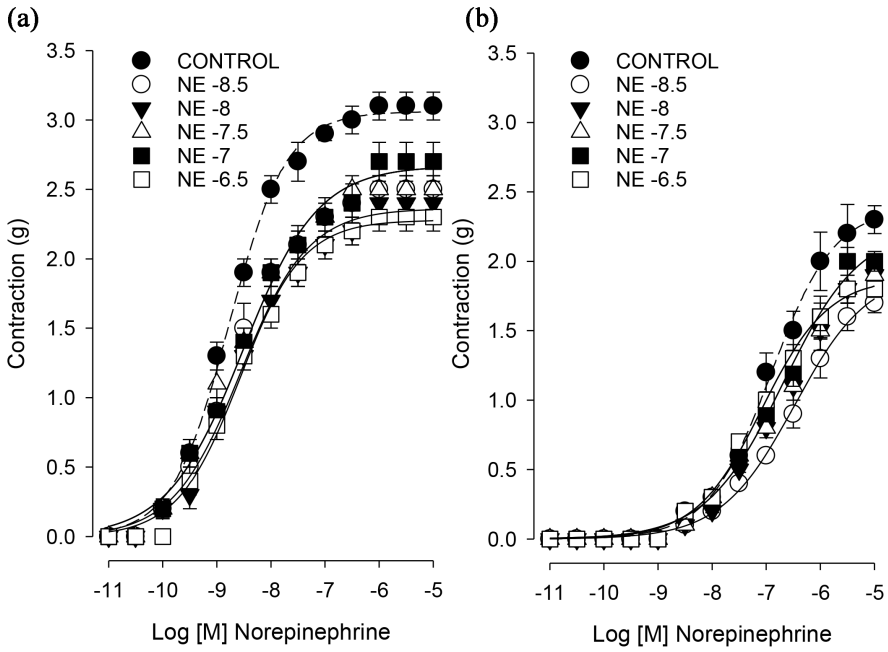

Figure 3. Concentration-response curves to norepinephrine (NE) in the aorta incubated in DMEM, for either 2 h (a) or 24 h (b) with different concentrations of added NE. (a) The dotted line with (●) signifies the NE-induced contraction in aorta following a 2 h incubation, referencing the control in Figure 2. (b) Similarly, the dotted line with (●) indicates the NE-induced contraction in the aorta after 24 h incubation, corresponding to the control in Figure 2. n = 8 rats.

efficacy in response to norepinephrine. However, the potency of the agonist remained unchanged at each interval (Table 1). Notably, the pD_2 for NE was high

in 2 h incubation (8.62) and lower after a 24 h incubation (6.64), denoting its affinity for α_{1D}-AR and $\alpha_{1A/B}$-AR, respectively.

To discern which α_1-AR mediated the action of norepinephrine at the two incubation times, we employed highly selective antagonists: 5-methylurapidil (5-MU, α_{1A}-AR), and BMY-7378 (BMY, α_{1D}-AR). As shown in Figure 4, incubating with different concentrations of 5-MU slightly shifted the CRC to the

Table 1. pD_2 values derived from tissues incubated for either 2 h or 24 h at 37˚C with varying concentrations of norepinephrine (ranging from 0 (control) and NE $1 \times 10^{-8.5}$ M to NE $1 \times 10^{-6.5}$ M, in half-log increments). The tissues were subsequently tested using a concentration-response curve (CRC) with norepinephrine. NE = norepinephrine, n = 8 rats.

Incubation in DMEM at 37˚C	2 h pD_2 (Mean ± S.E.)	24 h pD_2 (Mean ± S.E.)
Control (without NE)	8.73 ± 0.11	6.74 ± 0.13
NE $1 \times 10^{-8.5}$ M	8.63 ± 0.50	6.36 ± 0.09
NE $1 \times 10^{-8.0}$ M	8.78 ± 0.33	6.53 ± 0.06
NE $1 \times 10^{-7.5}$ M	8.80 ± 0.47	6.67 ± 0.10
NE $1 \times 10^{-7.0}$ M	8.68 ± 0.29	6.65 ± 0.10
NE $1 \times 10^{-6.5}$ M	8.14 ± 0.61	6.91 ± 0.12
Average	**8.62 ± 0.38**	**6.64 ± 0.11**

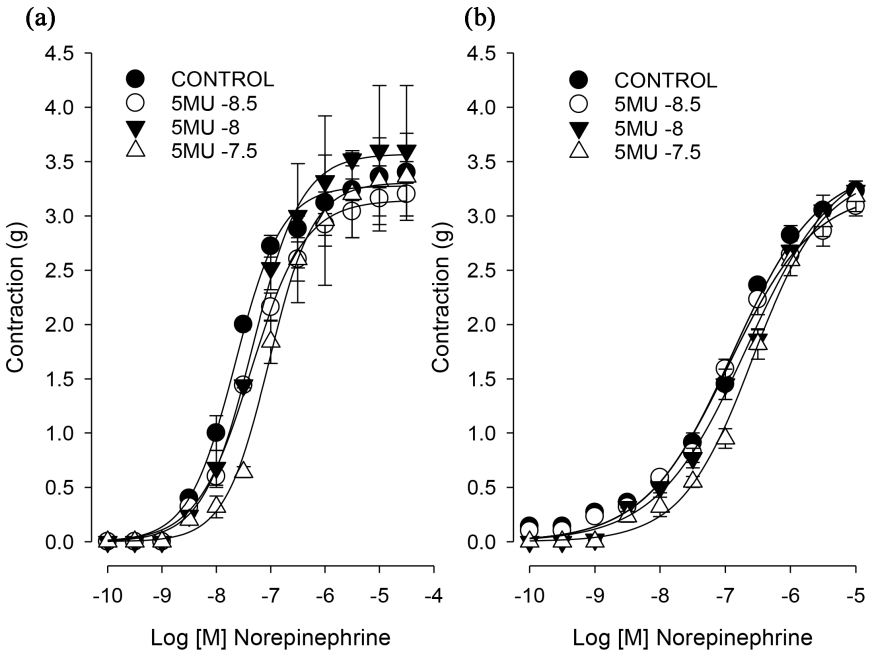

Figure 4. Concentration-response curves to norepinephrine (NE) in aorta incubated in DMEM for 2 h and 24 h with increased concentrations of α_{1A}-AR antagonist, 5-MU. (a) Representation of NE-induced contraction after 2 h incubation is marked with (●) for the following 5-MU $1 \times 10^{-8.5}$ M (○), 5-MU 1×10^{-8} M (▼), 5-MU $1 \times 10^{-7.5}$ M (△). (b) NE-induced contraction following a 24 h incubation is denoted with (●), 5-MU $1 \times 10^{-8.5}$ M (○), 5-MU 1×10^{-8} M (▼), 5-MU $1 \times 10^{-7.5}$ M (△). n = 8 rats.

right in response to NE after 2 h of incubation (pA_2 = 7.46, Figure 4(a)). In contrast, after 24 h of incubation, the CRC for norepinephrine was shifted to the right with a reduced maximal effect, and 5-MU exhibited minimal rightward shift, suggesting α_{1A}-AR was not primarily activated by NE (pA_2 = 6.91, Figure 4(b)). Conversely, a 2 h incubation followed by NE-induced contraction, antagonized with BMY-7378 caused a rightward shift in the CRC, signifying that the α_{1D}-AR predominantly mediates contraction in this vessel (pA_2 = 8.3, Figure 5(a)). A 24 h incubation, however, diminished the maximal contraction to NE, and BMY-7378 did not significantly shift the CRC rightward (pA_2 = 7.4, Figure 5(b)). In another experiment set, we examined the influence of the α_{1B}-AR alkylating antagonist CEC on the aorta incubated for 24 h; introducing CEC 40 min before NE stimulus completely abolished the contractile response (data not shown).

Seeking to uncover the underlying reason for the observed desensitization due to varied incubation duration (2 h *vs.* 24 h), the aorta was incubated with BMY-7378 for 24 h. At the end of this period, upon antagonist removal and subsequent NE challenge, the tissue's response to the adrenergic agonist was identical to the control curve. This suggests that endogenous norepinephrine, potentially released by the nerve endings in the *vasa vasorum*, but it had no desensitizing action, presumably due to α_{1D}-AR's protection by BMY-7378 (Figure 6).

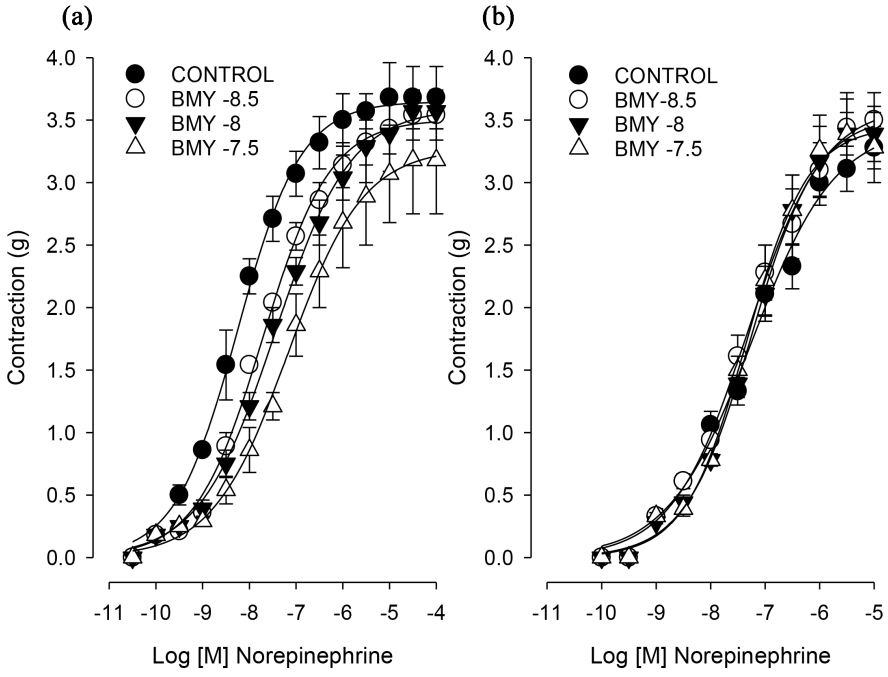

Figure 5. Concentration-response curves to norepinephrine (NE) in aorta incubated in DMEM for 2 h and 24 h, subjected to varying concentrations of the α_{1D}-AR antagonist, BMY-7378. (a) Representation of NE-induced contraction following a 2 h incubation is marked with (●), and for the subsequent BMY-7378 concentrations: BMY $1 \times 10^{-8.5}$ M (○), BMY 1×10^{-8} M (▼), BMY $1 \times 10^{-7.5}$ M (△). (b) NE-induced contraction after a 24 h incubation is denoted with (●). The responses in the presence of different BMY-7378 concentrations are represented as: BMY $1 \times 10^{-8.5}$ M (○), BMY 1×10^{-8} M (▼), BMY $1 \times 10^{-7.5}$ M (△). n = 8 rats.

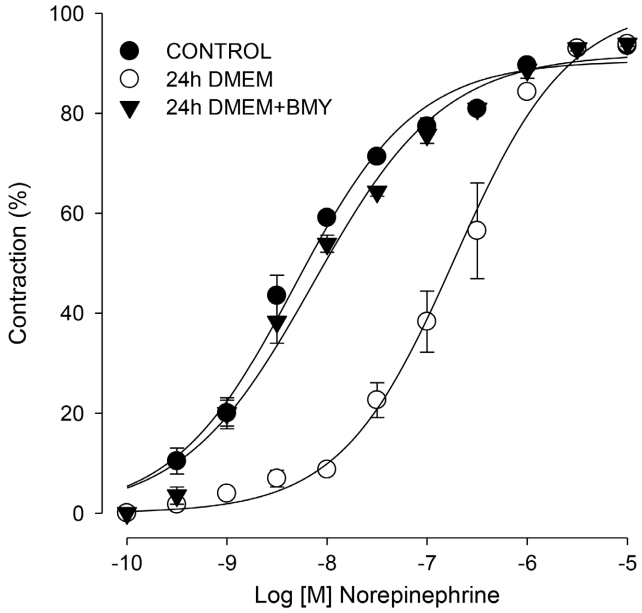

Figure 6. Concentration-response curves to norepinephrine (NE) in aorta incubated for 24 h in DMEM, both in the absence or the presence of BMY-7378 (10 μM). The NE-induced contraction in the aorta without any incubation is denoted by (●) and serves as the control curve. Contractions following 24 h of incubation are marked with (○), while responses after a 24 h incubation in the presence of BMY-7378 (10 μM) are represented by (▼). n = 8 rats.

4. Discussion

The α_1-adrenergic receptors (α_1-ARs) constitute a subfamily of GPCRs that are ubiquitously distributed throughout the organism. Upon stimulation by catecholamines, epinephrine and norepinephrine, they execute several functions. Chronic sympathetic activation of these receptors results in the contraction of vascular beds, thereby playing an integral role in modulating peripheral vascular resistance and blood pressure [32] [33]. Among the various α_1-ARs, α_{1D}-AR has been identified as the predominant receptor responsible for the contraction of large blood vessels, including the aorta and the carotid [25] [27]. Additionally, the α_{1D}-AR's phosphorylation and desensitization have been investigated in the context of norepinephrine-induced (homologous desensitization), and phorbol ester and tyrosine kinases action (heterologous desensitization) in transfected Rat-1 fibroblasts [2] [10] [19]. It also serves as a model for studying inverse agonism due to its notable overexpression and constitutive activity [34] [35] [36] [37].

Our findings unambiguously demonstrate that endogenously released norepinephrine from nerve endings in the *vasa vasorum* leads to vascular α_{1D}-AR desensitization (a form of homologous desensitization). This desensitization is circumvented when tissues are incubated with the α_{1D}-AR antagonist BMY-7378 over time. In contrast, many studies have reported α_1-ARs desensitization resulting from the addition of agonists, mimicking both homologous (e.g. norepinephrine, epinephrine, phenylephrine, oxymetazoline) and heterologous desen-

sitization (e.g. phorbol ester, and growth factors stimulating tyrosine kinases receptors) [2] [10] [19] [20] [24].

The observed pD_2 for NE after a 24 h incubation (6.74), is noteworthy. It suggests that the receptor, which is activated by norepinephrine under these incubation conditions (and thus desensitized), is not antagonized by 5-MU (an α_{1A}-AR antagonist). This finding is unexpected, especially considering that in the α_{1D}-AR null mouse model, the α_{1A}-AR assumes the contractile function in the aorta [38]. Therefore, besides α_{1D}-AR desensitization due to endogenous norepinephrine, an unresolved question persists: Which α_1-AR subtype mediates adrenergic-induced contraction in cultured aorta? Although the alkylating α_{1B}-AR antagonist CEC abolished the contractile response to NE, in aortas incubated for 24 h (data not shown), we suggest that another receptor (perhaps α_{1B}-AR or α_{1L}-AR) or a distinct mechanism could be implicated [39] [40]. In support of this, research indicates that even though α_{1A}-AR and α_{1B}-AR display similar densities in neonatal cardiomyocytes, the function of α_{1B}-AR is not known, particularly since its blockade with CEC amplifies the role of α_{1A}-AR in these cells [41]. Hence, additional experiments are needed to elucidate which α_1-AR is activated by norepinephrine under desensitized conditions.

5. Conclusion

Our study reveals that endogenous norepinephrine induces desensitization of the α_{1D}-AR in the aorta. However, this desensitization can be mitigated by the selective antagonist BMY-7378. It still needs to determine which specific α_1-AR remains functional after 24 hours of aorta incubation. Further studies are necessary to solve this enigma and provide a better understanding of the mechanisms involved.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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