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Original article

Influence of autonomic nervous system on relaxant action of bisphenol A in porcine myometrium

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Abstract

The mechanism of action of bisphenol A (BPA) in the myometrium has not been fully understood, which is why an attempt was made to determine the involvement of the key mechanisms (cholinergic, adrenergic and non-cholinergic/non-adrenergic) that regulate the uterine contractile activity in the relaxant effect of this BPA in cyclic gilts. Strips of myometrium were incubated for 15 min with the Krebs-Ringer solution (K-Rs), acetylcholine (ACh), atropine, epinephrine, phentolamine, bupranolol, sodium nitroprusside (SNP), methylene blue (MB) or N-ω-nitro L-arginine methyl ester hydrochloride (L-NAME), and then incubated for 15 min with increasing (10^{-8}–10^{-2} M) BPA concentrations. BPA administered at concentrations of 10^{-5}–10^{-2} M, 10^{-3}–10^{-2} M and 10^{-2} M after incubation with K-Rs, SNP and ACh and L-NAME, respectively, significantly reduced the uterine tension compared to the period before treatment. The amplitude of contractions was significantly reduced in myometrium pretreated with SNP and thereafter stimulated with BPA at concentrations of 10^{-3}–10^{-2} M and pretreated with K-Rs, ACh, epinephrine, phentolamine, L-NAME and MB, and stimulated with BPA at a concentration of 10^{-2} M compared to the period before treatment. BPA administered after incubation with ACh, atropine and SNP significantly reduced the frequency of uterine contractions at concentrations of 10^{-7}–10^{-2} M, after epinephrine and bupranolol at concentrations of 10^{-6}–10^{-2} M, after MB at concentrations of 10^{-5}–10^{-2} M, after K-Rs and phentolamine at concentrations of 10^{-3}–10^{-2} M, and after L-NAME at a concentration of 10^{-2} M compared to the period before treatment. BPA at concentrations of 10^{-4}-10^{-2} M significantly reduced the AUC value after prior administration of atropine, SNP and L-NAME, and at a concentration of 10^{-2} M after incubation with K-Rs, ACh, epinephrine, phentolamine, bupranolol and MB compared to the period before treatment. The results indicate that BPA's mechanism of action in the porcine myometrium is complex and that the final response to BPA's action results from multiple overlapping mechanisms of action. The autonomic system may slightly modify the action of BPA, with the nitrergic mechanism appearing to perform a more important role but with the guanyl cyclase/c-GMP mechanism being omitted.

Keywords: adrenergic, bisphenol A, cholinergic, gilts, myometrium, nitrergic, non-cholinergic/non-adrenergic, uterine contractility

Introduction

Bisphenol A (BPA) is an organic compound belonging to phenols. It was first described by Dianin in 1891 and synthesised by Zincke in 1905 from phenol and acetone (Brunelle 2005). This substance is widely used in the production of plastics and epoxy resins. BPA is present in many everyday items, such as food and beverage containers, thermal paper, dental products and toys (Vandenberg et al. 2007). BPA penetrates living organisms through the skin, digestive and respiratory tracts. Food, water and various elements of the environment are the main sources of BPA in humans (Michałowicz 2014). The legislation of the majority of countries established the dose of 50 µg/kg body weight/day as a tolerable daily intake for humans. However, the European Food Safety Authority temporarily decreased this dose to 4 µg/kg body weight/day (Almeida et al. 2018). Makowska et al. (2022) showed that in the loin meat of pigs who received capsules with BPA at a dose of 50 µg/kg body weight/day for 28 days, the levels of this BPA were insignificantly higher than in control animals (47.44 ± 4.39 vs 37.03 ± 6.18 ng/g dry weight). These results indicate that pork meat might be an important source of human intoxication with BPA. In addition, the presence of BPA in muscles indicates that this xenobiotic taken in with food, water and from the environment is distributed in the pig body and may affect various tissues/organs, including the reproductive system.

In the reproductive tract, exposure to BPA causes changes in the uterus, manifested by an increase in the thickness of the endometrium and the number of fibroblasts, as well as inhibition of the apoptotic process (Olson et al. 2017). These changes are accompanied by disorders of the estrous cycle (Nah et al. 2011), disturbances of embryo implantation after fertilisation (Yuan et al. 2018) and disorders of the uterine contractile activity (Gupta and Deshpande 2018, Zygmuntowicz et al. 2022). Exposure to BPA can also cause endometriosis (Louis et al. 2013) or cervical cancer (Ma et al. 2015).

It has been suggested that BPA can interact with many hormone receptors, including estrogen receptors (ERs), orphan receptor human estrogen-related receptor gamma (ERRγ), peroxisome proliferator-activated receptor gamma (PPARγ), androgen receptor (AR), glucocorticoid receptor (GR) and G protein-coupled oestrogen receptor (GPER) (Murata and Kang 2018, Buoso et al. 2020) and thus can cause dysfunction in many organs (Nahar et al. 2015) and may be involved in the development of hormone-sensitive cancers (Masi et al. 2021). For this reason, BPA is categorised as an endocrine disruptor (Beausoleil et al. 2018). A study conducted on sows that received estradiol-17β on the vaginal mucosa during artificial insemination found increased amplitude and duration of uterine contractions (Willenburg et al. 2004). The authors of those studies suggest that estradiol, through increased release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the uterus, causes increased contractility of the uterine myometrium. Thus, the mechanism leading to myometrial contraction after BPA administration may not be dependent only on estrogen receptor stimulation because available data indicate that exposure to BPA causes relaxation of the uterus (An et al. 2013, Salleh et al. 2015, Gupta and Deshpande 2017, Gupta and Deshpande 2018, Zygmuntowicz et al. 2022).

Uterine contractility is regulated by complex interactions between many factors related to the autonomic nerve system (Taneike et al. 1995), endocrine and auto/paracrine regulations (Cao et al. 2002, Kitazawa et al. 2003, Markiewicz et al. 2012) and intracellular and extracellular Ca^{2+} (Malik et al. 2021). It is generally accepted that contractions of the porcine myometrium can be stimulated by ACh (Kitazawa et al. 1999), oxytocin (OT) (Kitazawa et al. 2001a, Dittrich et al. 2009), $PGF_{2\alpha}$ (Mueller et al. 2006, Dittrich et al. 2009), leukotrienes (Jana et al. 2015), histamine (Kitazawa et al. 1997), endothelin (Isaka et al. 2000) and neuropeptide Y (Markiewicz et al. 2003). In contrast, the relaxation is caused by norepinephrine (NA), nitric oxide (NO) (Buxton 2004) and serotonin (Kitazawa et al. 2001b). These factors influence smooth muscle contractility directly or indirectly by affecting the synthesis and release of other substances.

Adequate uterine contractility is important for many reproductive functions (sperm transportation, embryo transport and implantation, pregnancy and parturition). Many xenobiotics, including BPA, may alter the contractile activity of the porcine uterus (Zygmuntowicz et al. 2022) and consequently could lead to adverse effects on fertility. BPA has been shown to reduce the amplitude and frequency of spontaneous uterine contractions in rats during the estrous phase through the involvement of mechanisms mediated by the cholinergic system (Salleh et al. 2015, Gupta and Deshpande 2017), NO (Gupta and Deshpande 2018), and the $PGF_{2\alpha}$ and OT pathway (An et al. 2013, Salleh et al. 2015). The authors' previous results also indicated that BPA at high concentrations reduced tension, and the amplitude and frequency of contractions of the porcine uterine myometrial strips collected from immature, cyclic (on days 12-14 of the oestrous cycle) and early pregnant (on days 12-16 of pregnancy) pigs (Zygmuntowicz et al. 2022), although the exact mechanism of this action has not been examined.

In the presented study, the involvement of the autonomic nerve system (cholinergic, adrenergic and

Table 1. Tested compounds and their concentration and action.

Tested compounds	Concentrations (moles)	Action	Reference
Acetylcholine	10^{-6}	cholinergic receptor agonist	Sarkar et al. 2016
Atropine	10^{-6}	cholinergic/muscarinic receptor antagonist	
Epinephrine	10^{-5}	adrenergic receptor agonist	
Phentolamine	10^{-5}	α-adrenergic receptor antagonist	
Bupranolol	10^{-5}	non-selective β_1-, β_2 – and β_3-adrenoceptor antagonist	
sodium nitroprusside	10^{-6}	nitric oxide donor	
methylene blue	10^{-4}	guanylyl cyclase inhibitor	
N-ω-nitro L-arginine methyl ester	10^{-4}	nitric oxide synthesis inhibitor	

non-cholinergic/non-adrenergic) in the mechanism of BPA relaxing action in the porcine myometrium was examined. A pig model was used for the study because it is applied for the examination of toxicokinetic (TK) properties of bisphenols (Gayrard et al. 2019) as a relevant species for investigating oral TK in humans (Kararli 1995). Moreover, the pig model is commonly used in research on the reproductive tract. The study used myometrial strips collected in the luteal phase, when progesterone is the predominant hormone, determining the development and maintenance of pregnancy (Likszo et al. 2021).

Materials and Methods

Reagents

Inorganic salts [sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4)] needed for the preparation of Krebs-Ringer solution (K-Rs) and glucose were purchased from Chempur (Piekary Śląskie, Poland). BPA, sodium nitroprusside (SNP), methylene blue (MB), N-ω-nitro L-arginine methyl ester hydrochloride (L-NAME), phentolamine hydrochloride, epinephrine, acetylcholine chloride, atropine sulphate and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Bupranolol was purchase from LGC Standarts GmbH (Wesel, Germany). BPA stock solution (10^{-1} M) was prepared in DMSO, and serial dilutions were made with deionised water on the day of the experiment. A stock solution (10^{-2} M) was prepared in deionised water, and final dilutions were made in K-Rs for the remaining tested substances.

Animals

The research was conducted on mixed gilts (Large White x Polish Landrace; n=8) with a body weight of 110-120 kg, intended for commercial slaughter and meat processing. The phase of the estrus cycle was confirmed by ovarian morphology (corpora lutea attained maximal mass, were "liver coloured" and very vascular, corpora albicantia were barely visible, and follicles had initial growth) (Akins and Morrissette 1968). The uteri were collected immediately after slaughter at the meat processing plant ("TOMUS" Tomasz Reihs, Królikowo, Poland) and transported on ice to the laboratory within 0.5 hours. In accordance with Polish (Anonymous 2015) and European (Anonymous 2010) regulations on the protection of animals used for scientific or educational purposes, the experiments did not require the consent of the relevant bioethics committee for animal experiments.

Preparation of uterine strips and measurement of their contraction

Myometrial strips (3 × 5 mm) were collected as previously described (Jana et al. 2013, Markiewicz et al. 2016), washed with saline solution and mounted between two stainless steel hooks in a 5 mL Schuler Organ Bath type 809 (Hugo Sachs Electronic, Germany) under a resting tension of 10 mN. The strips were kept in K-Rs with the following composition [millimoles per liter (mM/L)]: NaCl -120.3, KCl -5.9, $CaCl_2$ -2.5, $MgCl_2$ -1.2, NaH_2PO_4 -1.2, $NaHCO_3$ -15.5, glucose - 11.5 and pH 7.4. The solution was maintained at 37°C and continuously saturated with 95% O_2 and 5% CO_2. Uterine smooth muscle contractile activity was measured using a force transducer (HSE F-30 type 372) with a type 570 bridge coupler, while graphic recording was performed on a Hugo Sachs Elektronik recorder using PowerChart software (AD Instruments, Australia). Recording of contractions was started after a 60-90 minute preincubation period. To check the tissue viability and suitability for testing, the sections were stimulated with increasing (10^{-6}-10^{-4} M) concentrations of ACh. After washing, the strips were incubated for 15 min with K-Rs (control) or with the substances presented in Table 1 and then stimulated with increasing

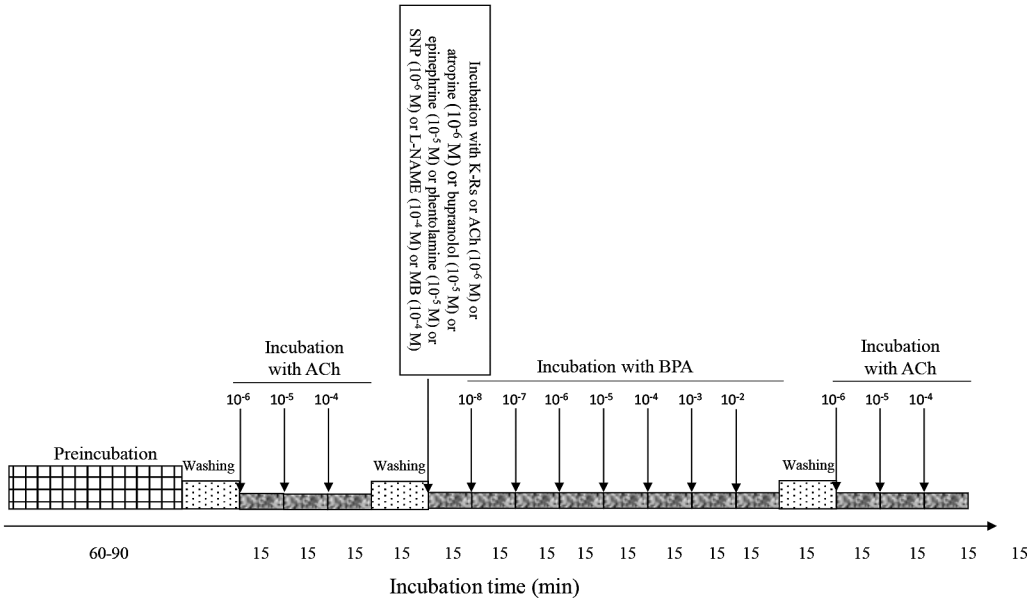

Fig. 1. Diagram showing the chronology of stimulation of the porcine uterine strips. K-Rs – Krebs-Ringer solution; SNP – sodium nitroprusside; MB – methylene blue; L-NAME – N-ω-nitro L-arginine methyl ester; ACh – acetylcholine; BPA – bisphenol A. The concentrations of the substances used are given in moles (M).

(10^{-8}-10^{-2} M) concentrations of BPA added at 15-minute intervals. Myometrial strips collected from eight pigs were used to study the effect of each examined substance.

Doses of SNP, MB, L-NAME, bupranolol, phentolamine, epinephrine, ACh and atropine were determined based on Sarkar et al. (2016) and BPA based on Zygmuntowicz et al. (2022). The sections were then washed and stimulated again with ACh at concentrations of 10^{-6}-10^{-4} M. Only results with a difference in response to ACh stimulation at the beginning and end of the experiment of less than 20% were included in the statistical analysis. The scheme of the experiment is shown in Fig. 1.

Statistical analysis

Parameters for spontaneous contractile activity of the myometrial strips [tension - changes in resting tension (the preload force which is set by stretching the muscle) expressed in milinewtons (mN), amplitude - the developed force between the baseline and maximum peak expressed in mN, and frequency – the number of observed contraction peaks and area under the curve (AUC) measured by calculating the integral of the appropriate section of the curve] were calculated using LabChart software (LabChart 8, AdInstruments, Colorado Springs, CO, USA) for 15 minutes before administration (before treatment) of K-Rs or tested substances and taken as 100%. Results calculated for 15-minute periods after the administration of K-Rs or the tested substances were expressed as percentages of the uterine tension, amplitude and frequency of contractions and AUC value measured during the period before stimulation. Using Bonferroni's multiple comparisons test, the statistical significance of differences between pre- and poststimulation periods was assessed by one-way ANOVA (GraphPad Prism 6.07, GraphPad Software, San Diego, CA, USA). Differences with a p value below 0.05 were considered significant.

Concentration-effect curves were fitted to the best selected model. Selection was made based on the Akaike criterion value according to the following equation: $Y=Bottom + (Top-Bottom)/(1+(X/EC_{50}))$ where Y is the analysed effect level, EC_{50} is the tested substance concentration which produces a 50% measured effect, and X is the tested active substance concentration. The top and Bottom are plateaus in units of the Y axis used last squares regression with weighting $1/Y^2$, and outliers analysis by Rout test Q=10% were implemented. The EC_{50} values were then calculated from the best fit of the concentration-response curve from the raw data (GraphPad Prism 10.0.3). Differences among mean rank values of groups were compared using 1-way ANOVA without matching or pairing, using a nonparametric Kruskal-Wallis test, and Dunn's correction for multiple comparisons (GraphPad Prism 10.0.3). Differences with a p value below 0.05 were considered statistically significant.

Results

The example diagrams showing contractile activity of the myometrial strips after stimulation with the examined substances are presented in Fig. 2.

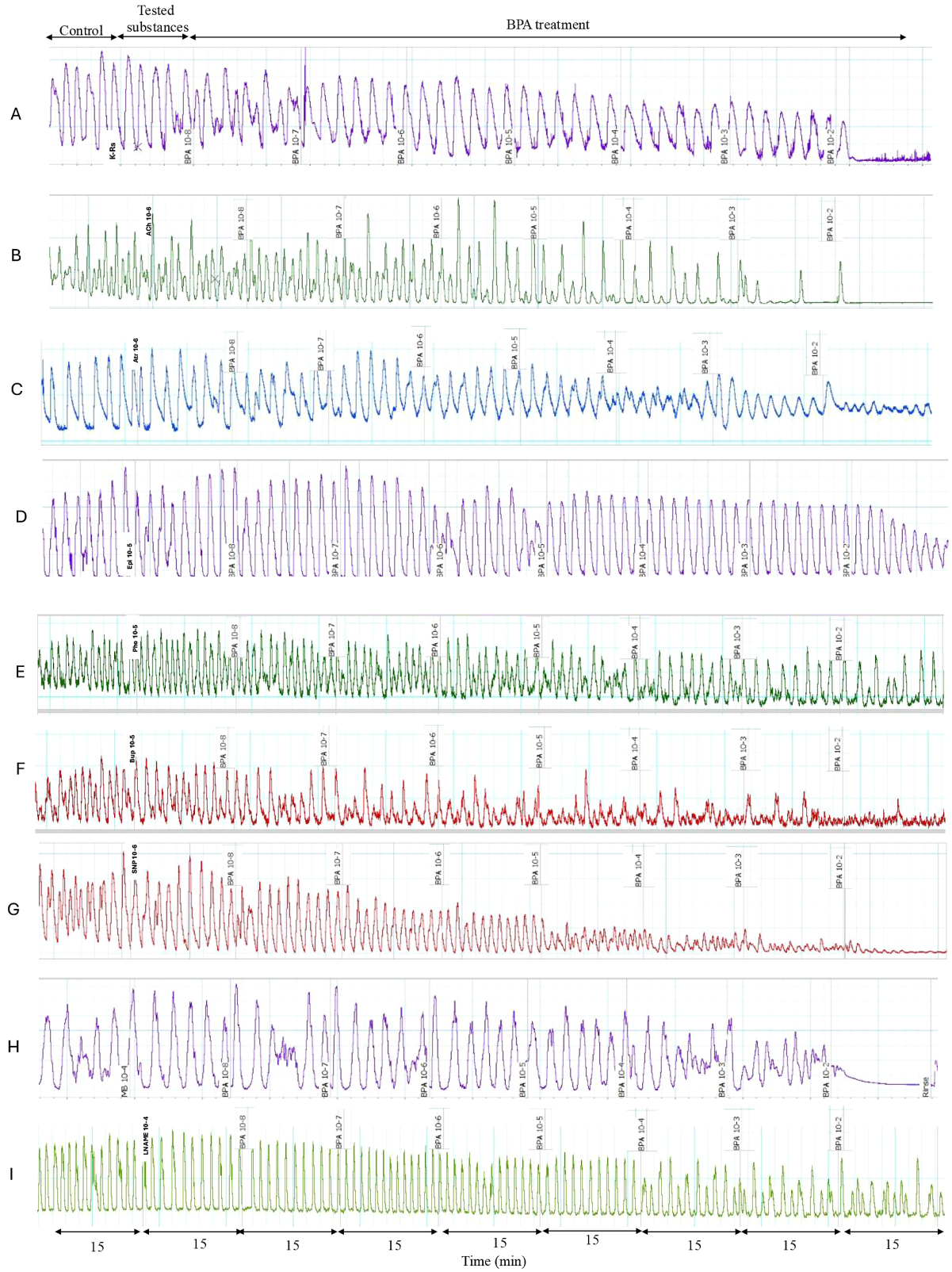

Fig. 2. Example diagrams showing the contractile activity of the porcine uterine strips collected from cyclic pigs (on day 12-14 of the oestrous cycle) exposed for 15 min to (A) Krebs-Ringer solution (K-Rs), (B) acetylcholine (ACh), (C) atropine (Atr), (D) epinephrine (Epi), (E) phentolamine (Phe), (F) bupranolol (Bup), (G) sodium nitroprusside (SNP), (H) methylene blue (MB) or (I) N-ω-nitro L-arginine methyl ester hydrochloride (L-NAME) and then stimulated with increasing (10^{-8} –10^{-2} M) bisphenol A (BPA) concentrations.

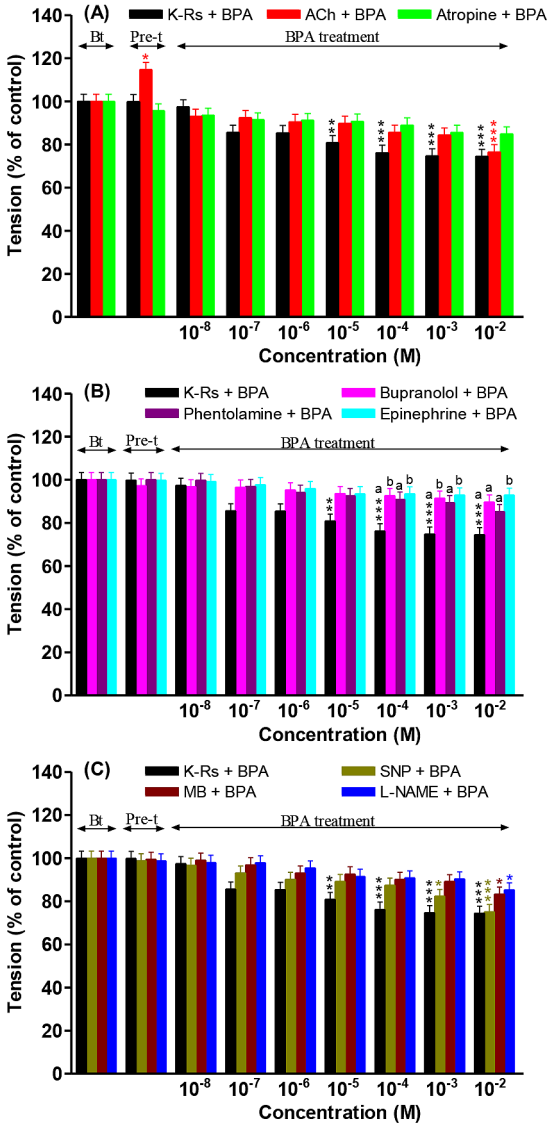

Fig. 3. Changes in the tension of the porcine myometrial strips (collected on days 12-14 of the estrous cycle) following stimulation with (A) Krebs-Ringer solution (K-Rs), acetylcholine (ACh, 10^{-6} M) or atropine (10^{-6} M), (B) K-Rs, epinephrine (10^{-5} M), phentolamine (10^{-5} M) or bupranolol (10^{-5} M) and (C) K-Rs, sodium nitroprusside (SNP; 10^{-6} M), N-ω-nitro L-arginine methyl ester (L-NAME, 10^{-4} M) or methylene blue (MB, 10^{-4} M), and then increasing concentrations of bisphenol A (BPA, 10^{-8}–10^{-2} M). The results calculated for 15 minutes following the administration of each test substance were expressed as a percentage (mean ± SD; n=8) of the values observed for 15 minutes before treatment (Bt). Pre-t - time of action of the test substances before administering BPA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ – statistically significant differences compared to the Bt period; ^{a,b} – letters mark the statistically significant difference for the same BPA concentration.

Effect of BPA following preincubation with K-Rs, ACh, atropine, epinephrine, phentolamine, bupranolol, SNP, L-NAME or MB on the myometrium tension

ACh administered before BPA caused a significant increase in the tension compared to the period before treatment. The other tested substances (K-Rs, atropine, epinephrine, phentolamine, bupranolol, SNP, L-NAME and MB) did not cause significant changes in tension compared to the period before treatment (Fig. 3).

BPA administered after K-Rs, SNP and ACh, L-NAME or MB significantly reduced the tension at concentrations of 10^{-5}–10^{-2} M, 10^{-3}–10^{-2} M and 10^{-2} M, respectively compared to the period before treatment. Moreover, no significant changes were observed after prior administration of atropine, epinephrine, phentolamine and bupranolol (Fig. 3). BPA administered after K-Rs at concentrations of 10^{-4}-10^{-2} M and 10^{-4}-10^{-3} M caused a significantly greater decrease in the tension than that after epinephrine ($p<0.05$-$p<0.01$) and bupranolol ($p<0.05$), respectively. However, EC_{50} analysis of BPA did not show any statistically significant differences between groups (Table 2).

Table 2. Summary of mean (± SD) EC_{50} values for bisphenol A (BPA)-mediated myometrial relaxation in the presence of Krebs-Ringer solution (K-Rs), acetylcholine (ACh), atropine, epinephrine, phentolamine, bupranolol, sodium nitroprusside (SNP), N-ω-nitro L-arginine methyl ester (L-NAME) or methylene blue (MB).

Tested Substances	Parameter			
	Tension	Amplitude	Frequency	AUC
K-Rs + BPA	$2.96\times10^{-4} \pm 5.10\times10^{-4}$	$3.19\times10^{-4} \pm 4.52\times10^{-4}$	$8.11\times10^{-4} \pm 7.42\times10^{-4}$	$2.16\times10^{-3} \pm 2.35\times10^{-3}$
ACh +BPA	$4.21\times10^{-6} \pm 5.59\times10^{-6}$	$1.18\times10^{-5} \pm 1.35\times10^{-5}$	$2.26\times10^{-6} \pm 2.10\times10^{-6}$	$1.34\times10^{-4} \pm 1.22\times10^{-4}$
Atropine + BPA	$6.55\times10^{-5} \pm 14.2\times10^{-5}$	$5.53\times10^{-4} \pm 13.3\times10^{-4}$	$2.40\times10^{-6} \pm 3.52\times10^{-6}$	$8.44\times10^{-6} \pm 12.8\times10^{-6}$
Epinephrine + BPA	$2.66\times10^{-5} \pm 3.38\times10^{-5}$	$1.90\times10^{-4} \pm 3.11\times10^{-4}$	$3.11\times10^{-5} \pm 3.82\times10^{-5}$	$6.07\times10^{-6} \pm 8.00\times10^{-6}$
Bupranolol + BPA	$9.83\times10^{-6} \pm 8.66\times10^{-6}$	$1.61\times10^{-5} \pm 3.49\times10^{-5}$	$2.59\times10^{-6} \pm 3.61\times10^{-6}$	$4.95\times10^{-5} \pm 11.4\times10^{-5}$
Phentolamine + BPA	$2.15\times10^{-5} \pm 1.41\times10^{-5}$	$4.98\times10^{-6} \pm 9.70\times10^{-6}$	$2.38\times10^{-3} \pm 4.97\times10^{-3}$	$3.76\times10^{-4} \pm 4.52\times10^{-4}$
SNP + BPA	$1.70\times10^{-4} \pm 3.37\times10^{-4}$	$7.35\times10^{-4} \pm 8.20\times10^{-4}$	$1.02\times10^{-4} \pm 2.06\times10^{-4}$	$3.49\times10^{-3} \pm 5.09\times10^{-3}$
MB + BPA	$2.43\times10^{-5} \pm 4.14\times10^{-5}$	$7.49\times10^{-4} \pm 12.1\times10^{-4}$	$1.57\times10^{-3} \pm 1.58\times10^{-3}$	$2.00\times10^{-5} \pm 3.47\times10^{-5}$
L-NAME + BPA	$8.16\times10^{-4} \pm 11.1\times10^{-4}$	$6.29\times10^{-5} \pm 6.60\times10^{-5}$	$11.7\times10^{-3} \pm 17.1\times10^{-3}$*	$2.43\times10^{-3} \pm 1.62\times10^{-3}$

* $p<0.05$, when compared with K-Rs + BPA

Effect of BPA following preincubation with K-Rs, ACh, atropine, epinephrine, phentolamine, bupranolol, SNP, L-NAME or MB on the uterine contraction amplitude

A significant increase in the amplitude was observed after the administration of epinephrine and phentolamine compared to the period before treatment (Fig. 4). The increase in the amplitude was significantly greater following the administration of epinephrine ($p<0.01$) and phentolamine ($p<0.05$) compared to K-Rs.

BPA administered after SNP significantly reduced the amplitude at concentrations of 10^{-3}–10^{-2} M, and when administered after K-Rs, ACh, epinephrine, phentolamine, L-NAME and MB, BPA significantly reduced the amplitude at a concentration of 10^{-2} M, with no effect after the administration of bupranolol and atropine compared to the period before treatment (Fig. 4). BPA at a concentration of 10^{-2} M caused a significantly lower ($p<0.05$) decrease in the amplitude after bupranolol and a significantly greater decrease ($p<0.001$) after SNP than that after the administration of K-Rs. EC_{50} analysis of BPA did not show any statistically significant differences between groups (Table 2).

Effect of BPA following preincubation with K-Rs, ACh, atropine, epinephrine, phentolamine, bupranolol, SNP, L-NAME or MB on the uterine contraction frequency

No statistically significant changes were noted in the frequency of contractions following a 15-minute preincubation with the test substances compared to the period before treatment (Fig. 5).

BPA administered after ACh, atropine and SNP significantly reduced the uterine contraction frequency at concentrations of 10^{-7}–10^{-2} M, after epinephrine and bupranolol at concentrations of 10^{-6}–10^{-2} M, after MB at concentrations of 10^{-5}–10^{-2} M, after KR-s and phentolamine at concentrations of 10^{-3}–10^{-2} M, and after L-NAME at a concentration of 10^{-2} M compared to the period before treatment (Fig. 5). BPA at a concentration of 10^{-5} M caused a significantly greater ($p<0.001$) reduction in frequency of contractions after prior administration of ACh, atropine and SNP compared to K-Rs. At a concentration of 10^{-4} M caused a significantly greater ($p<0.001$) decrease after prior administration of ACh, atropine, bupranolol and SNP. At a concentration of 10^{-3} M caused a significantly greater decrease after prior administration of ACh, atropine, bupranolol, SNP ($p<0.001$) and epinephrine ($p<0.05$). At a concentration of 10^{-2} M caused a significantly higher ($p<0.001$) reduction in the frequency after prior administration of all examined substances compared to K-Rs (Fig. 5.). EC_{50} analysis of BPA showed a statistically significant difference prior administration of L-NAME compared to K-RS (Table 2).

Effect of BPA following preincubation with K-Rs, ACh, atropine, epinephrine, phentolamine, bupranolol, SNP, L-NAME or MB on the AUC value

A significant increase in the AUC value was observed after the administration of atropine, phentolamine ($p<0.05$) and epinephrine ($p<0.01$) compared to the period before treatment (Fig. 6).

BPA at concentrations of 10^{-4}–10^{-2} M significantly reduced the AUC value after prior administration of atropine, SNP and L-NAME, and at a concentration of 10^{-2} M after K-Rs, ACh, epinephrine, bupranolol, MB and phentolamine compared to the period before treatment (Fig. 6). BPA at a concentration of 10^{-2} M caused a significantly greater ($p<0.001$) reduction in the

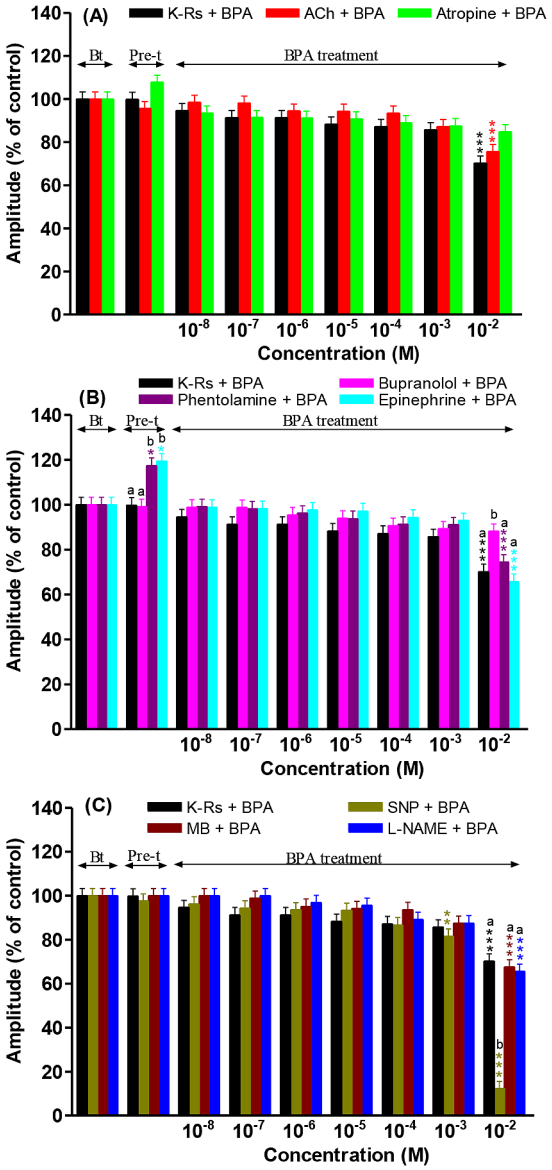

Fig. 4. Changes in the contraction amplitude of the porcine myometrial strips (collected on days 12-14 of the estrous cycle) following stimulation with (A) Krebs-Ringer solution (K-Rs), acetylcholine (ACh, 10^{-6} M) or atropine (10^{-6} M), (B) K-Rs, epinephrine (10^{-5} M), phentolamine (10^{-5} M) or bupranolol (10^{-5} M) and (C) K-Rs, sodium nitroprusside (SNP; 10^{-6} M), N-ω-nitro L-arginine methyl ester (L-NAME, 10^{-4} M) or methylene blue (MB, 10^{-4} M), and then increasing concentrations of bisphenol A (BPA, 10^{-8}–10^{-2} M). The results calculated for 15 minutes following the administration of each test substance were expressed as a percentage (mean ± SD; n=8) of the values observed for 15 minutes before treatment (Bt). Pre-t – time of action of the test substances before administering BPA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ – statistically significant differences compared to the Bt period. ^{a,b} – letters mark the statistically significant difference for the same BPA concentration.

AUC value after prior administration of SNP and L-NAME compared to K-Rs ($p<0.001$). EC_{50} analysis of BPA did not show any statistically significant differences between groups (Table 2).

Discussion

The results indicate that BPA administered after K-Rs reduced the tension, amplitude and frequency of contractions and the AUC value only at high concentrations. The direction of the changes observed was consistent with the authors' previous study, in which a significant inhibitory effect of BPA on the tension as well as the amplitude and frequency of contractions in cyclic pigs was observed after the application of high concentrations of this bisphenol (Zygmuntowicz et al. 2022). The observed relaxant effect of BPA in pigs was also consistent with the results obtained for cats (Kabakçi et al. 2019) and rats (An et al. 2013, Gupta and Deshpande 2017, Gupta and Deshpande 2018). However, higher concentrations of BPA were required to induce a relaxant effect in the pigs. An et al. (2013) also demonstrated that high-dose BPA exposure

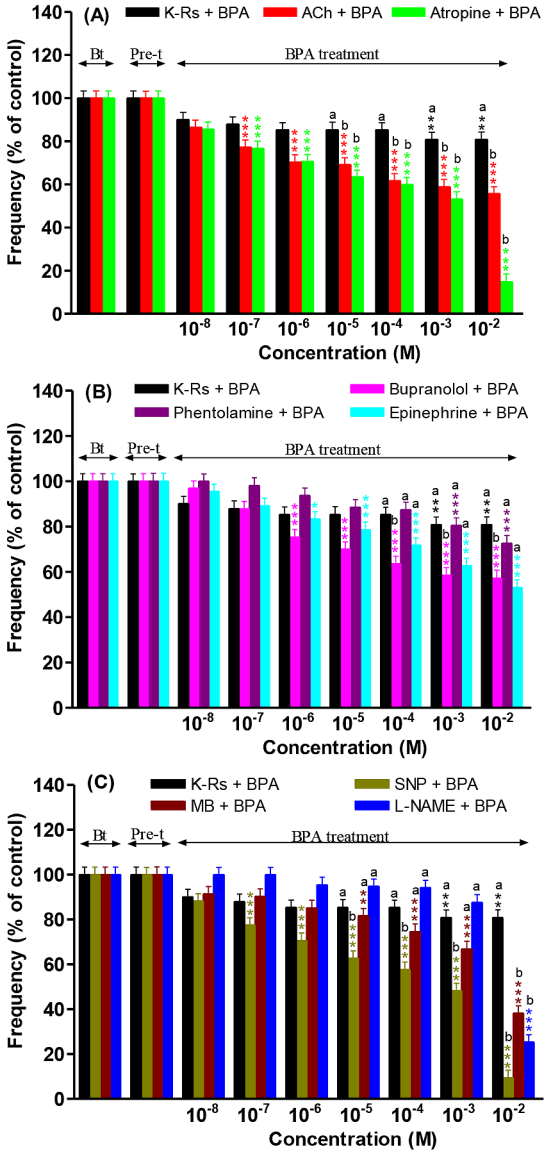

Fig. 5. Changes in the contraction frequency of the porcine myometrial strips (collected on days 12-14 of the estrous cycle) following stimulation with (A) Krebs-Ringer solution (K-Rs), acetylcholine (ACh, 10^{-6} M) or atropine (10^{-6} M), (B) K-Rs, epinephrine (10^{-5} M), phentolamine (10^{-5} M) or bupranolol (10^{-5} M) and (C) K-Rs, sodium nitroprusside (SNP; 10^{-6} M), N-ω-nitro L-arginine methyl ester (L-NAME, 10^{-4} M) or methylene blue (MB, 10^{-4} M), and then increasing concentrations of bisphenol A (BPA, 10^{-8}–10^{-2} M). The results calculated for 15 minutes following the administration of each test substance were expressed as a percentage (mean ± SD; n=8) of the values observed for 15 minutes before treatment (Bt). Pre-t – time of action of the test substances before administering BPA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ – statistically significant differences compared to the Bt period. ^{a,b} – letters mark the statistically significant difference for the same BPA concentration.

decreased uterine contractility and altered transcript and protein levels of contraction-associated factors (increased oxytocin and oxytocin receptor and decreased prostaglandin $F_2\alpha$ receptor) in rats. The above study results indicate that BPA has a relaxant effect on the myometrium, with the uterine sensitivity to the action of BPA differing in the individual animal species and being determined by the physiological conditions (sexual maturity and the phase of the cycle and pregnancy).

In regulating uterine contractions, the autonomous system serves an important role. Kitazawa et al. (1999) concluded that both exogenous and endogenous ACh causes, already at low concentrations, a contraction of the porcine myometrium. Markiewicz et al. (2016) also observed a significant increase in both uterine tension and contraction frequency and a reduction in the amplitude of contractions, both in cyclic and pregnant pigs, after the administration of ACh. Taneike et al. (1994) observed that, in response to ACh, the contractile intensity of the longitudinal muscle isolated from non-pregnant uteri of gilts was the most potent in the horns, slightly weaker in the corpus and weakest in the cervix. In contrast, in the circular muscle, contractile responses

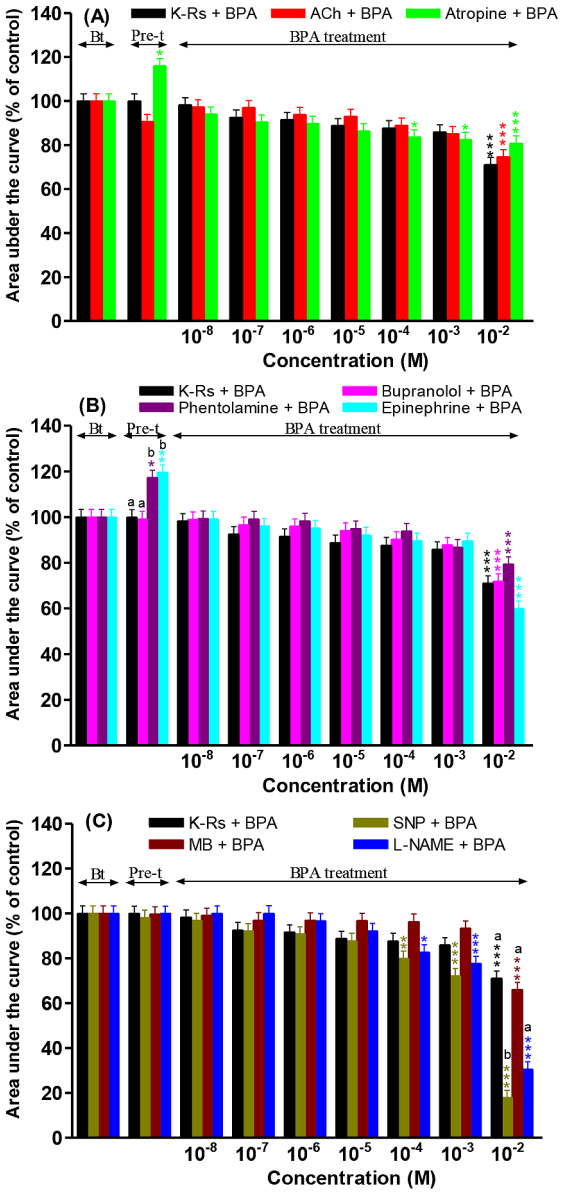

Fig. 6. Changes in the value of the area under the curve (AUC) following the stimulation of the porcine myometrial strips (collected on days 12-14 of the estrous cycle) following stimulation with (A) Krebs-Ringer solution (K-Rs), acetylcholine (ACh, 10^{-6} M) or atropine (10^{-6} M), (B) K-Rs, epinephrine (10^{-5} M), phentolamine (10^{-5} M) or bupranolol (10^{-5} M) and (C) K-Rs, sodium nitroprusside (SNP; 10^{-6} M), N-ω-nitro L-arginine methyl ester (L-NAME, 10^{-4} M) or methylene blue (MB, 10^{-4} M), and then increasing concentrations of bisphenol A (BPA, 10^{-8}–10^{-2} M). The results calculated for 15 minutes following the administration of each test substance were expressed as a percentage (mean ± SD; n=8) of the values observed for 15 minutes before treatment (Bt). Pre-t – time of action of the test substances before administering BPA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ – statistically significant differences compared to the Bt period. ^{a,b} – letters mark the statistically significant difference for the same BPA concentration.

elicited by ACh indicated no regional variation. This data indicates that the type of myometrial tissue tested may also determine the response to cholinergic stimulation. In this study, ACh significantly increased the tension without affecting the other test parameters. BPA, administered after incubation with ACh, caused a significant reduction in the tension, amplitude of contractions, and in the AUC value only at the highest concentration; however, the frequency of contractions was reduced for the BPA applied across a wide range of concentrations. In addition, BPA administered after ACh at concentrations of 10^{-5}–10^{-2} M resulted in a significantly greater reduction in contraction frequency than BPA administered after K-Rs. The results obtained show that the stimulation of muscarinic receptors does not block the relaxant effect of BPA. However, after blocking the muscarinic receptors with atropine, it was demonstrated that, at the concentration applied, it caused no significant changes in the test parameters. The results of the current study are consistent with those of previous studies, which demonstrated that the administration of atropine does not affect the myometrial tonus

or the frequency and amplitude of spontaneous contractions (Taneike et al. 1994, Kitazawa et al. 1999). The results obtained in this study indicate that BPA administered after atropine caused a significant reduction in the frequency of contractions and AUC value. Moreover, BPA administered after atropine caused a significantly greater reduction in the frequency of contractions compared to its administration after K-Rs. The results show that blocking the muscarinic receptors intensifies the action of BPA used at high concentrations with regard to inhibiting the frequency of porcine myometrial contractions. However, the results also show that neither the stimulation nor the blocking of the muscarinic receptors significantly changes the direction of the BPA action, which may suggest that the cholinergic system is not involved in the direct mechanism of the action of BPA.

The adrenergic system also regulates uterine contractile activity, and it is documented that epinephrine suppresses uterine contractions (Dušić et al. 2024, Tumanova et al. 2004,). In the current study, epinephrine caused an increase in the amplitude and the AUC value. It should be noted that the expression of all isoforms of α_1- and α_2-adrenergic receptors, and all subtypes of β-adrenergic receptors, was revealed in the myocytes of the myometrium in animals, and the activation of α-adrenergic receptors stimulates myometrial contractility. In contrast, the activation of β-adrenergic receptors decreases the contractility (Jana and Całka 2023). In our study BPA administered after epinephrine at concentrations of 10^{-6}-10^{-2} M reduced the frequency of contractions, whereas only at the highest concentration did it reduce the amplitude and the AUC value. In addition, BPA at concentrations of 10^{-3}-10^{-2} M, administered after epinephrine, resulted in a greater reduction in contraction frequency than that for BPA administered after K-Rs. The results show that the stimulation of adrenergic receptors with epinephrine affects, to the greatest extent, the frequency of contractions, with this effect not significantly altering the relaxant action of BPA.

The blocking of α-adrenergic receptors with phentolamine caused no significant changes in the tension and frequency of contractions, but an increase was observed in the amplitude and the AUC value. In contrast, Taneike et al. (1994) demonstrated that the transmural stimulation in the presence of phentolamine attenuated the contractions of the cornual longitudinal muscle isolated from non-pregnant uteri of gilts. In addition, the current data differ from the results of a study by Fanning et al. (2017), which demonstrated a slight effect of prazosin (α_1 – adrenergic antagonist), yohimbine (α_2 – adrenergic antagonist) on myometrial contractility, and a small reduction in myometrial contractions, caused by phentolamine in myometrial strips obtained from women undergoing elective caesarean delivery. However, it should be noted that since these results were obtained from a small number of myometrium samples (n=4) collected from women in the final stages of pregnancy and not from cyclic pigs, the extrapolation of this data is not very credible. In the presented study, BPA administered at high concentrations after phentolamine caused a significant reduction in the amplitude and frequency of contractions and the AUC value, with this effect not being significantly different from the action of BPA administered after K-Rs. The obtained results show that blocking the α-adrenergic receptors has no significant effect on the action of BPA.

Bupranolol, which blocks the β-adrenergic receptors, caused no significant changes in the test parameters. A similar lack of a significant effect of this β-blocker was observed in the porcine myometrium collected from the gilts on days 12-14 of pregnancy (Markiewicz and Jaroszewski 2016). Another study showed that bupranolol attenuated contractions in the myometrial strips obtained from women undergoing hysterectomy for benign gynecological disorders and in endometrial and cervical cancers, whereas opposite effects were observed in ovarian and synchronous ovarian-endometrial cancers (Modzelewska et al. 2021). The authors indicate that ovarian cancer considerably alters the contractile activity of the nonpregnant human uterus in response to β-adrenoceptor antagonists. However, it is difficult to relate these results to the current study due to the different materials used to study myometrial contractile activity. In the current study, BPA administered after bupranolol caused a significant reduction in the frequency of contractions (at concentrations of 10^{-6}–10^{-2} M) and the AUC value (at the highest concentration). Moreover, BPA administered after bupranolol only at high concentrations caused a significantly greater reduction in the frequency of contractions compared to its administration after K-Rs, which suggests that β-adrenergic regulation plays no crucial role in the action of BPA.

It has been suggested that BPA decreased the amplitude and frequency of spontaneous uterine contractions in rats showing oestrous phase by involving the nitrergic mechanism (Gupta and Deshpande 2018). In the current study, SNP, a nitric oxide donor, caused no significant changes in the test parameters. A similar no-relaxant effect on the spontaneous contractions of the myometrium from pregnant rats was observed by Hennan and Diamond (1998) after the application of SNP at a concentration of 5 mM. On the other hand, Hoffmann et al. (2003) demonstrated that increasing concentrations of SNP induced a concentration-dependent inhibition of the phasic contractile activity of

human myometrial strips collected from women undergoing hysterectomy for dysfunctional uterine bleeding. The above results indicate considerable differences in the myometrial sensitivity to the action of SNP, depending on the type of tissue used in the study, which hinders the possibility of comparing the results obtained in different units. The current study demonstrated that BPA administered after SNP caused a significant reduction in all parameters examined. In addition, BPA administered after SNP caused a significantly greater reduction in the frequency of contractions at concentrations of 10^{-5}–10^{-2} M and in the amplitude and the AUC value at a concentration of 10^{-2} M, compared to its administration after K-Rs. These results indicate that SNP intensifies the relaxing effect of BPA, especially concerning the frequency of contractions.

The administration of L-NAME caused no significant changes in the test parameters. Similarly, Gupta and Deshpande (2018) did not observe a significant effect of L-NAME on the basal tone, amplitude and frequency of spontaneous uterine contractions in rats. In the current study, BPA administered at the highest concentration after L-NAME caused a significant reduction in the tension, amplitude and frequency of contractions and the AUC value. In turn, Gupta and Deshpande (2018) demonstrated that L-NAME blocked the BPA-induced decrease in amplitude at all concentrations but antagonised the frequency only at the maximum BPA concentration (10 μM). Despite the slight differences observed between the present study results, they indicate that the nitrergic mechanism is involved in the relaxant action of BPA.

In the current study, MB, a guanylyl cyclase inhibitor, caused no significant changes in the test parameters. These results differed from the data obtained by Modzelewska and Kostrzewska (2021), who demonstrated that MB applied at the same concentration increases the AUC value and the frequency of contractions of the human non-pregnant myometrium. In contrast, the results of the current study are consistent with those of Gupta and Deshpande (2018), who demonstrated that MB applied at the same concentration as in the current study does not cause a significant alteration in the amplitude or frequency of spontaneous contractions or the basal tone. In the current study, BPA administered after MB caused a significant reduction in the frequency and, in high concentrations, the amplitude and AUC value. BPA administered after MB, only at the highest concentration, caused a significantly greater reduction in the frequency of contractions than the administration after K-Rs. The results of the current study are consistent with those obtained in a study of the rat myometrium (Gupta and Deshpande 2018). The above results show that the inhibiting guanylyl cyclase activity does not contribute significantly to a change in BPA action.

In conclusion, the mechanism of action of BPA in the porcine myometrium is complex, and the final effect of BPA is the result of multiple overlapping mechanisms of action. The results obtained in the current study show that the autonomic system may slightly modify the action of BPA, with the nitrergic mechanism appearing to perform a more important role but with the guanyl cyclase/c-GMP mechanism being omitted.

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