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Characterization of in vitro relaxant mechanisms in erectile tissue from rabbits of different ages

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Abstract In the present study we investigated the in vitro relaxant response of erectile tissue obtained from rabbits of different ages (3, 7 and 24 months) in order to detect the progression with age of cavernosal activity in response to substances acting via endothelium-dependent or -independent mechanisms. Noradrenaline induced a concentration-dependent contraction (0.1 μM–3 mM), with an increase in the contractility in the 24-month-old group. Acetylcholine produced a concentration-dependent relaxant effect in the three age groups, with a reduction of the maximal relaxant effect in older animals. ATP (10 μM–1 mM) and adenosine (10 μM–1 mM) induced a concentration-dependent relaxant effect that was higher in the older group. The presence of the NO-synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (0.1 mM) or of the P_2-purinoceptor antagonist suramin did not affect ATP relaxation. Relaxation induced by sodium nitrite and nifedipine was reduced in older animals. In conclusion, aging selectively alters the in vitro responsiveness of rabbit erectile tissue. Purinergic system remains more active despite a decrease in the maximal endothelial cholinergic activity and the direct smooth muscle relaxant component.

Key words Erectile tissue · Ageing · Purines · Acetylcholine · Sodium nitrite · Nifedipine

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Introduction

Erection is produced by dilatation of arterioles perfusing lacunar spaces in the corpus cavernosum and by relaxation of the surrounding smooth muscle, which causes engorgement with blood. Compression of the outflow venules against the tunica albuginea induces a reduction of blood outflow, leading to a rise in intracavernosal pressure [1, 34]; a mathematical model for the complex phenomenon of penile erection has also been suggested [37]. Impairment of the mechanisms that support relaxation of corpus cavernosum smooth muscle may lead to impotence. Erectile dysfunction is a major clinical pathological condition in adult men and a frequent cause is altered vascular responsiveness [19, 28]. Numerous studies have evaluated the role of possible mediators of cavernosal smooth muscle relaxation, such as acetylcholine [39], vasoactive intestinal polypeptide [2] and prostaglandins [21]. More recently, nitric oxide (NO) has been suggested as a primary physiological relaxing substance [27], particularly influencing non-adrenergic non-cholinergic (NANC) neurotransmission [9, 31]. Adenosine and adenine nucleotides, released from nerve endings, are able to modulate penile tissue responses [11], and have even been proposed as hypothetical therapeutic agents for impotence. Therefore, great interest has been shown in the role of endogenous modulators of smooth muscle relaxation [26], which may contribute to the pathophysiology of erection and impotence, particularly in diabetes mellitus [29, 36] or with age-related abnormalities of smooth muscle fibres [5]. The use of animal models can be particularly helpful for detecting time-dependent evolution of penile responsiveness. In the present study we studied the in vitro contractile and relaxant responses of corpus cavernosum obtained from rabbit of different ages, in order to detect the evolution with age of responses of corpus cavernosum induced by molecules acting via endothelium-dependent or -independent mechanisms.

Material and methods

Male New Zealand White rabbits of three different ages were used: 3 months of age ($n = 9$, 2–2.2 kg, young), 7 months of age ($n = 9$, 3–3.5 kg, adult) and 24 months ($n = 9$, 4–5 kg, old). The choice of the age groups was based on our previous experience in aortic tissue functionality [13, 14] and on other published studies [42]. Principles of laboratory animal care were followed. The rabbits were fed with a cholesterol-free standard rabbit diet (2RB15-GLP, Italiana Mangimi, Milano, Italy). The mortality rate of aged rabbits did not exceed 15% and was due to complications from pulmonary infections. There was approximately 100% complete copulatory behaviour in 7-month-old rabbits, which declined to about 40% in 24-month-old animals.

Animals were anaesthetized with urethane (2 mg/kg i.p.) and sacrificed by cervical dislocation. The penis was removed at the point of attachment to the ischium, placed in saline solution (see later) at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2. The erectile tissue was dissected free from the tunica albuginea and from each animal four strips were obtained. One preparation was used to determine the cumulative concentration-response curve of the contractile agent noradrenaline. Each relaxant drug was tested on five strips, each from different animals. The preparation was suspended in a 30-ml tissue bath containing modified Krebs-bicarbonate solution of the following composition (mmol/l): NaCl 116.0; KCl 3.2; $CaCl_2$ 1.2; $MgCl_2$ 1.2; NaH_2PO4 1.2; $NaHCO_3$ 22.0; glucose 10.1; ascorbic acid 1.1, and equilibrated with a 95% O_2–5% CO_2 gas mixture, pH 7.4, at 37 °C.

Isometric tension was recorded by means of force transducers (Type DY0 Basile, Comerio, Italy) connected to a chart recorder (Unirecord Basile, Comerio, Italy). The preparation was held at a resting tension of 20 mN and allowed to equilibrate at optimal length for 60–90 min before experiments were started, the buffer being changed every 15 min. Preparations were pre-contracted with the approximate EC_{50} of noradrenaline (30 μM), washed and equilibrated for at least 60 min before experiments were started. This procedure was found to increase and stabilize any subsequent contractile response to noradrenaline. One preparation was used to determine the cumulative concentration-response curve of noradrenaline in order to calculate the EC_{50} (concentration inducing half-maximal contraction) of the contractile agonist for each rabbit. For relaxation studies, vasodilator drugs were cumulatively added to preparations precontracted with EC_{50} noradrenaline to steady-state tension. At the end of the experiments, EC_{50} noradrenaline was added to verify contraction stability. The spontaneous relaxation following this contraction was subtracted from the relaxation caused by the vasodilator agonists in the previous curve, to calculate the net effect of the agonists. Of the different relaxing drugs that were tested, in our experimental conditions, prostaglandin E_1 (PGE_1) and the calcium ionophore A23187 failed to produce any relaxant effect in the preparation precontracted with NA.

Drugs and reagents

Adenosine 5′-triphosphate (ATP) sodium salt, adenosine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), acetylcholine (ACh) bromide, sodium nitrite, nifedipine, A23187 and noradrenaline (NA) bitartrate were purchased from Sigma (St. Louis, MO, USA). Suramin was kindly supplied by Dr. A. Faggiotto (Bayer, Milan, Italy). PGE_1 (alprostadil) was a kind gift from Upjohn, Caponago, Italy.

Statistical analysis

The percentage relaxation caused by each drug was calculated by assigning the maximal contraction at steady state induced by noradrenaline EC_{50} as 100%. Data were expressed as means ± SEM. Differences between means were compared by Student's two-tailed t-test for unpaired data, and a probability level of 0.05 was accepted as significant.

Results

Contraction by noradrenaline

On initial incubation in saline solution the preparations presented slight spontaneous contractions from baseline tone. The addition of NA induced a concentration-dependent contraction in the concentration range (10 nM–3 mM); the maximal contractions among the different groups of age were 12, 15 and 38 mN at 3, 7 and 24 months, respectively, with 3 mM NA. The differences were statistically significant between 3, 7 and 24 months (Fig. 1A); normalization of the data to the respective maximum values indicated different NA sensitivities between 3, 7 and 24 months (Fig. 1B).

Relaxation induced by acetylcholine

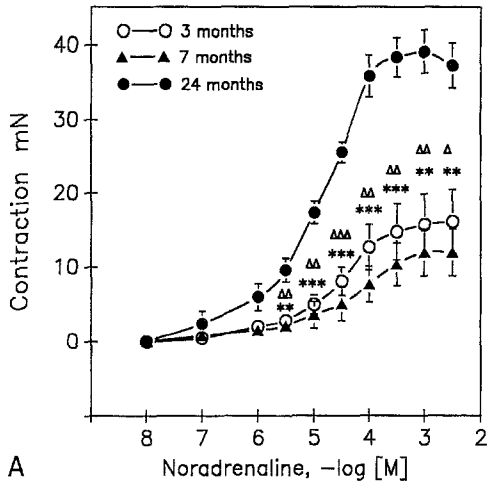
On NA-precontracted tissue, ACh produced a concentration-dependent relaxant effect in the three age groups (Fig. 2). However, 24-month-old animals showed a greater relaxant effect of ACh in the concentration range 0.1–1 μM, followed by a reduction of the maximal relaxant tone at higher concentrations. No significant variation was observed between the 3-month-old and 7-month-old animals (Fig. 2).

Relaxation induced by purines

ATP induced a concentration-dependent relaxant effect in the NA-precontracted preparations from all groups (Fig. 3). The relaxant effect obtained in aged animals was, however, significantly higher than that in the two younger groups. Twenty minutes preincubation of the preparations with the NO-synthase inhibitor L-NAME (0.1 mM) or with suramin (0.1 mM) did not affect the relaxation induced by ATP in any group (data not shown). Adenosine induced a concentration-dependent relaxation of the preparations (Fig. 4). The relaxant response was slightly but significantly higher in tissues from older animals treated with adenosine 0.1 mM.

Relaxation induced by sodium nitrite and nifedipine

The relaxation produced by sodium nitrite is presented in Fig. 6. While the preparations from the young rabbits were responsive to the drug, the relaxation in older



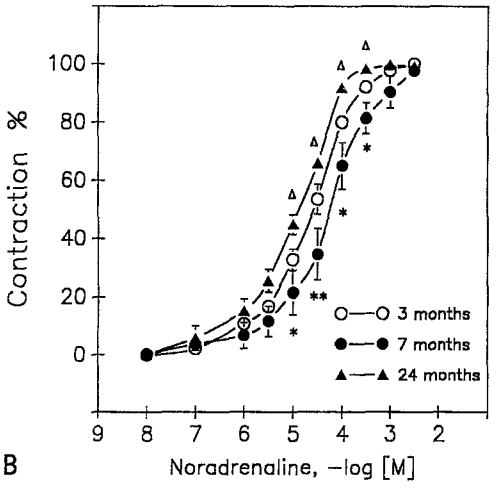


Fig. 1A,B Contraction induced by noradrenaline in erectile tissue obtained from rabbits of different ages. Responses are presented as direct force developed (mN, **A**) or percentage maximal response (**B**). Points are means ± SE obtained from nine strips, each from a different rabbit. △ $P < 0.05$, △△ $P < 0.01$ and △△△ $P < 0.001$, 3 months vs 24 months. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, 7 months vs 24 months

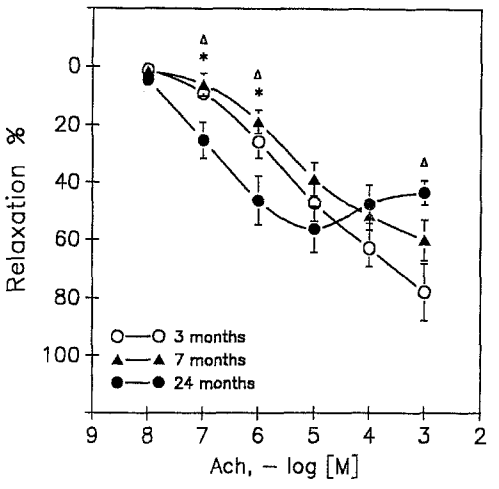


Fig. 2 Relaxing response to acetylcholine (*ACh*) of erectile tissue from rabbits of different ages. Points are means ± SE obtained from five strips, each from a different rabbit. △ $P < 0.05$, 3 months vs 24 months. * $P < 0.05$, 7 months vs 24 months

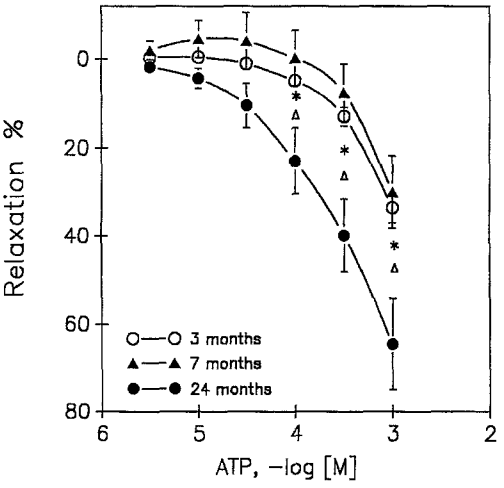


Fig. 3 Response to ATP of erectile tissue from rabbits of different ages. Points are means ± SE obtained from five strips, each from a different rabbit. △ $P < 0.05$, 3 months vs 24 months. * $P < 0.05$, 7 months vs 24 months

rabbits appeared to be reduced; responses in preparations from the 24-month-old group presented more than a 60% reduction in vascular relaxation at the 10-mM sodium nitrite concentration (Fig. 5). Another drug acting through endothelium-independent mechanisms is the calcium-channel blocker nifedipine. The drug induced a concentration-dependent relaxation that was reduced in older animals, but was significantly greater at the intermediate age (Fig. 6). No differences were found between 3 and 7 months of age.

Discussion

The present data demonstrate that rabbit erectile tissue in vitro undergoes age-related changes in sensitivity to drugs. In particular, the effects of the classical endothelium-dependent relaxant drug ACh are reduced in older animals at the higher concentration, while the effect of purinergic drugs appears to be enhanced. Conversely, the response to a calcium antagonist or to sodium nitrite is reduced in older animals.

Due to the difficulty of obtaining human penile erectile tissue, the use of cavernosal tissue from animals, especially rabbits, has been proposed for in vitro and in vivo studies [4]. It should be remembered that neurotransmission and cavernosal tissue responses to various drugs may show significant species differences. However, the present findings in rabbit erectile tissue may yield some information about changes due to ageing.

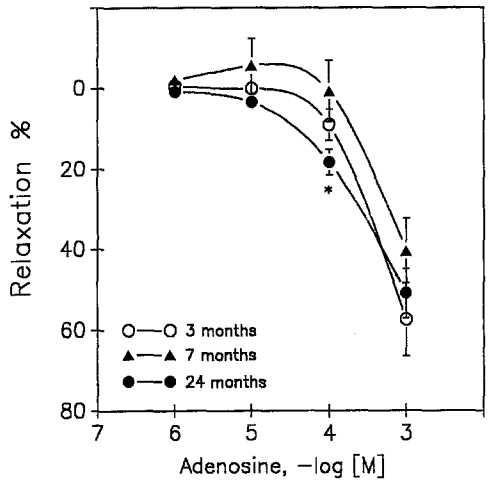


Fig. 4 Response to adenosine of erectile tissue from rabbits of different ages. Points are means ± SE obtained from five strips, each from a different rabbit. * $P < 0.05$, 7 months vs 24 months

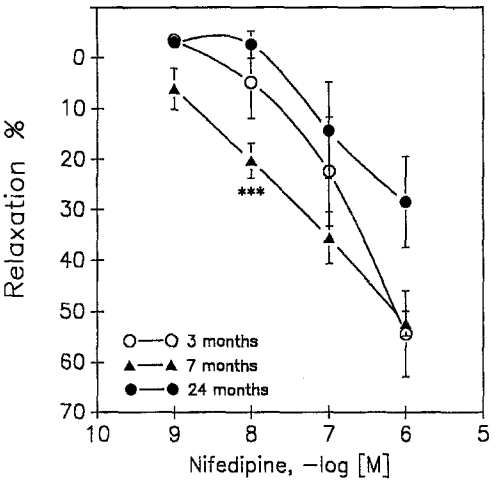


Fig. 6. Relaxing response to nifedipine of erectile tissue from rabbits of different ages. Points are means ± SE obtained from five strips, each from a different rabbit. *** $P < 0.001$, 7 months vs 24 months

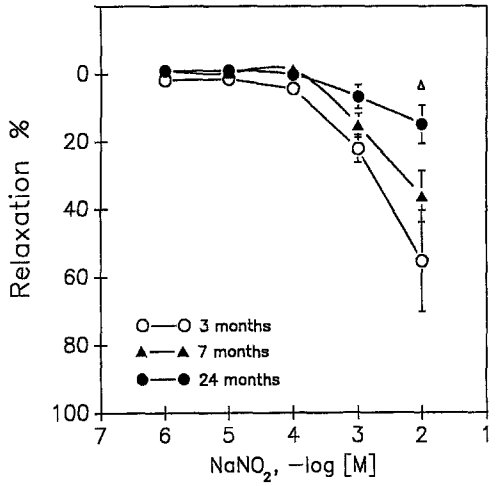


Fig. 5 Relaxing response to sodium nitrite of erectile tissue from rabbits of different ages. Points are means ± SE obtained from five strips, each from a different rabbit. △ $P < 0.05$, 3 months vs 24 months

Many authors have reported that various endogenous mediators or pharmacological agents are able to induce relaxation of cavernosal smooth muscle [3, 9, 11, 35]; among these are adenosine, ATP and prostaglandins. The non-adrenergic non-cholinergic system appeared to be particularly involved in the penile erectile response, since ATP has been shown to be able to induce cavernosal tissue relaxation [31, 41]. The present data suggest that ATP, as well as its metabolite adenosine, is specifically involved in the maintenance of corpus cavernosal activity in ageing. We report here that the cavernosal response to purines in old rabbits is well maintained and even augmented compared to young and adult rabbits, unlike the response to the cholinergic agonist ACh which is impaired in old animals. Lack of muscarinic receptor activity is considered as a precocious marker of endothelial impairment, such as in the atherosclerotic process [12, 18] or in ageing [22]. In accordance with previous observations from various vascular areas, in cavernous tissue muscarinic endothelial activity is altered in the elderly. Interestingly, despite a reduced maximal response to acetylcholine in older animals, the activity of the drug was increased at lower concentrations, suggesting a hypersensitivity of penile muscarinic receptors. The data obtained using other relaxants may indicate receptor targets for defining the pathophysiology of impotence and reduced penile responsiveness in ageing. The response to purines involves the recognition of their mechanism of action. ATP acts via purinergic P_2 receptors [7, 8]; in particular, P_{2y} purinoceptors have been identified in many vascular beds, mediating vasorelaxation by ATP [15, 32]. However, ATP can be hydrolysed by ectonucleotidases [5, 20, 25] and produce adenosine, which relaxes vessels via P_1 purinoceptors of the A_2 type, located both on vascular smooth muscle [16, 33] and on endothelium [16, 33, 44]. This mechanism of relaxation induced by adenosine and ATP has been suggested to exist in cavernosus tissue by Chiang et al. [11], who used antagonists of the A_{2b} purinoceptor type. In the present study, the lack of inhibition induced by suramin suggests that ATP-induced relaxation in corpus cavernosum from rabbits of the different ages is not due to P_{2y} purinoceptor activation. A P_1-mediated response could be hypothesized, according to Chiang et al. [11], maintaining the high degree of responsiveness in ageing. Our data further indicate that this effect appears not to be NO mediated, since L-NAME is not effective in altering ATP relaxation, suggesting that the receptor involved is located on smooth muscle. This result is in contrast with other studies which indicated that NO was responsible for the NANC

neurogenic endothelium-independent relaxation of corporal smooth muscle [23, 27, 31].

Intracavernosal injection of PGE_1 is currently used to treat impotence [10, 24, 38]. Increased synthesis of cAMP induced by PGE_1 has been reported in the penis of the diabetic rat, suggesting an upregulation of adenylate cyclase activity on E_1 receptor coupling as an adaptive phenomenon [29]. In accordance with other authors [40], PGE_1 did not induce any relevant relaxation of corporeal tissue in our experimental conditions (data not shown), confirming that rabbits are not susceptible to the action of this prostaglandin.

Responses to the calcium-channel blocker nifedipine suggested that ageing impairs the smooth muscle component related to voltage-sensitive calcium channels located in smooth muscle [30]. These results can be explained as a diffuse phenomenon of vascular ageing, already described in other areas, including the thoracic aorta [30]. The age-related reduction of cavernosal tissue relaxation to sodium nitrite also confirms that smooth muscle function is impaired. This finding is in agreement with decreased responses to papaverine and histological alterations of the structure of the corpora cavernosa mainly due to an increase in collagen fibres and a decrease in smooth muscle and elastic fibres [6, 10, 17, 43].

In summary, the present data lead to the conclusion that aging selectively alters the in vitro responsiveness of rabbit corpus cavernosum, as observed in other vascular areas [13, 14]. In rabbit erectile tissue, the purinergic system remains active despite decreases in maximal endothelial cholinergic activity and direct smooth muscle components.

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