A functional study of purinergic signalling in the normal and pathological rabbit corpus cavernosum

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Level of Evidence   3b

OBJECTIVE

To examine rabbit cavernosal smooth muscle (CSM) relaxation to ATP, ADP and UTP in normal rabbits and in models of conditions that predispose to erectile dysfunction (ED), diabetes mellitus (DM; induced for 6 months) and bladder outlet obstruction (BOO, 6 weeks after surgery).

MATERIALS AND METHODS

Concentration-response curves (CRCs) were constructed to ATP, ADP and UTP on CSM from control rabbits in the absence and presence of antagonists. In addition, CRCs were constructed to ATP in CSM from rabbits with DM and BOO.

RESULTS

ATP and UTP caused equipotent, dose-dependent relaxations of pre-contracted normal rabbit CSM; ADP was more potent. Relaxation was inhibited by Reactive Blue 2, but not by suramin, 8-p-sulfophenyltheophylline or L-N^G-nitroarginine methyl ester. In rabbits with DM and those with partial BOO, ATP-mediated CSM relaxation was less than in control rabbits. Pharmacological profiling suggests that purine-induced CSM relaxation might be mediated by P2Y$_1$ and P2Y$_4$ receptors in the rabbit.

CONCLUSIONS

In healthy rabbits, ATP released from nerves appears to produce relaxation of CSM via P2Y$_4$ receptors on smooth muscle, while ADP, acting on P2Y$_1$ receptors on endothelial cells, produces relaxation via nitric oxide. Alterations in CSM purinergic signalling might be implicated in the pathophysiology of ED associated with DM and BOO. Characterization of purinergic signalling in CSM might highlight new therapeutic targets for treating ED.

KEYWORDS

purinergic receptors, cavernosum, erectile dysfunction, diabetes mellitus, bladder outflow obstruction

INTRODUCTION

The flow of blood into the corpus cavernosum dictates the rigidity of the penis and is controlled by the cavernosal smooth muscle (CSM), which is contracted in the normal flaccid state. Attaining and maintaining a penile erection depends on the ability to adequately relax the CSM. The tone of the CSM can be altered by different neurotransmitters, but the local release of nitric oxide (NO) appears to be critical [1]. Alteration in the ability to control and relax the CSM leads to erectile dysfunction (ED). Importantly, ED can be treated by pharmacologically manipulating the CSM tone.

The purine ATP is a cotransmitter released by sympathetic and parasympathetic nerves, that can induce SM contraction or relaxation in a various organs via specific receptors [2,3].

Alterations in purinergic signalling have been implicated in the pathophysiology of vascular [4] and urological diseases [5]. ATP induces dilation of isolated canine and bovine penile arteries [6].

ATP concentration-dependently relaxes pre-contracted rabbit CSM strips [7]. In different vascular beds ATP can either relax vascular SM directly via postjunctional P2Y receptors, or indirectly via the stimulation of endothelial NO [4]. Rubbing the rabbit CSM strip to disrupt the endothelium (histologically confirmed) decreased nerve-mediated CSM relaxation, but had no effect on the ATP-induced relaxation [8]. ATP caused a concentration-dependent relaxation in pre-contracted human CSM strips [9]. The NO-synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) did not affect ATP-induced human CSM relaxation, supporting the earlier evidence that the response is endothelium-independent [10]. The nonspecific P2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid, which has some preference for P2X receptors in vivo [11], had no effect [10]. Adenosine-5′-O-(2-thiodiphosphate) (ADPβS; an active P2Y$_1$ agonist) caused relaxation in CSM strips taken from men with ED at the time of prosthesis implantation [12]. This action was blocked by the nonspecific P2 receptor antagonist, Reactive Blue 2 (RB2) which has some preference for P2Y receptors in vivo [11]. Unlike the ATP response, the ADPβS-mediated relaxations were reduced by 70–80% by physical disruption of the endothelium and by L-NAME [12]. P2Y$_1$ receptor mRNA has been detected by RT-PCR and Northern blotting in the rat penis, and in situ hybridization located the mRNA on endothelial cells of the corpus cavernosum [13]. In vivo studies found that an intracavernosal injection with ATP in dogs increased the intracavernosal pressure and

induced erection [14]. This effect was not due to changes in systemic blood pressure. Adenosine 5′-O-(3 thiotriphosphate) (ATPγS) induced penile erection in the cat [15].

The role that different neurotransmitters play in the pathophysiology of ED has been inferred from observed changes in neurotransmitter signalling in animal models of conditions showing ED. Some such evidence already exists; alterations in cavernosal purinergic signalling were reported in a rat model of diabetes mellitus (DM) [16], ageing rabbits [17], a rabbit model of chronic steroid abuse [18] and castrated rabbits [19].

The present study had three objectives: (i) A pharmacological study of normal rabbit CSM that attempted to determine the receptor subtypes mediating the ATP-induced CSM relaxation in the rabbit. Although ATP-mediated CSM relaxation has been studied in streptozocin-induced DM rats [16], functional purinergic characterization studies have not yet been reported in this species. The role that purinergic signalling plays in controlling erectile function has been much more extensively studied in the rabbit than the rat or human, although the rabbit model of DM has not yet been tested. (ii) To determine whether the ATP-mediated CSM relaxation is altered in the rabbit model of alloxan-induced DM. (iii) To test whether there is any alteration in purinergic signalling in the corpus cavernosum of rabbits with experimentally induced BOO. There is increasing evidence from human studies that BOO is an independent risk factor for ED [20–22]. Alterations in the expression of endothelin receptors were identified in the corpus cavernosum of a rabbit model of BOO [23], but other neurotransmitter systems have not yet been studied in this context.

MATERIALS AND METHODS

Breeding, maintenance and killing of the rabbits followed the principles of good laboratory animal care and experimentation, in compliance with UK Home Office regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986. DM was induced in a group of rabbits as follows: six age-matched (3 kg) male adult New Zealand White rabbits were given an i.v. injection of alloxan (65 mg/kg) via the ear

vein; six other age-matched rabbits were used as controls. The rabbits were fed *ad libitum* with standard rabbit plain diet (SDS, Witham, UK) and allowed free access to water. Serum glucose was estimated to confirm the induction of DM, and the experiments were conducted 6 months after the induction of DM.

BOO was surgically induced in six adult male New Zealand White rabbits (3 kg) as follows: general anaesthesia was induced using 1–2% halothane in O_2. An 8 F paediatric urethral catheter was inserted (Foley, CR Bard International Ltd, Crawley, UK). A lower midline laparotomy was made and the bladder base carefully mobilized, avoiding contact with all nerves. The rabbit bladder protrudes more into the peritoneal space than in the human, allowing this procedure to be done with minimal dissection or disruption of tissues. A 2/0 silk ligature was tied around the proximal urethra, the wound incision closed and the urethral catheter removed. The tension of the suture was carefully judged so that the rabbits could void after surgery, and had similar degrees of BOO. The rabbits recovered and were fed *ad libitum* with SDS standard plain diet and allowed free access to water. Six age- and weight-matched, sham-operated control rabbits had the same procedure, with the immediate removal of the silk ligature. Experiments were conducted 6 weeks after surgery.

Experiments to characterize the CSM from healthy adult (3 kg) New Zealand White rabbits were conducted; the rabbits were killed and their penises immediately excised, and placed in chilled Krebs' solution. CSM strips were dissected out and experiments carried out as detailed below.

To measure biochemical indices, blood was sampled at monthly intervals via the ear vein in DM and control rabbits, placed in serum gel bottles and serum sodium, urea, creatinine and glucose concentrations determined using standard methods on a Hitachi 717 Automatic Autoanalyser (Boehringer Mannheim, Lewes, Sussex). In the partial BOO rabbits and age-matched controls, blood was also sampled via the ear vein at 6 weeks and analysed as above.

For organ-bath studies, penile tissue was removed and placed in cold oxygenated Krebs' solution. The tunica albuginea was opened and the corpus cavernosum was dissected out and cut in strips of ≈1 × 1 × 5 mm. The size

and weights of the strips were similar throughout the studies. The strips were mounted vertically in organ baths and bathed in Krebs' solution of the following composition (mM): NaCl 133, KCl 4.7, NaHPO₄ 1.35, NaHCO₃ 16.3, MgSO₄ 0.61, glucose 7.8, CaCl₂ 2.52, pH 7.4, at 37 °C and aerated with 95% O_2/5% CO_2. Atropine (1 μM), guanethidine (5 μM) and indomethacin (10 μM) were added to inhibit the cholinergic, adrenergic and cyclooxygenase pathways. An initial tension of 2 g was applied to strips. Tension was subsequently measured by a force-displacement transducer (FT-03C, Grass Instruments, Quincy, MA, USA). Strips were equilibrated for ≥1 h and then challenged with KCl (124 mM). Reproducible contractions were consistently obtained.

Characterization experiments were carried out on CSM strips from control rabbits as follows: strips were pre-contracted with phenylephrine (100 μM), then relaxed with ATP alone (1 nM to 10 mM), or ATP in the presence of 8-*p*-sulfophenyltheophylline (8-*p*SPT, 10 μM; P1 receptor antagonist), suramin (100 μM; nonspecific P2 receptor antagonist), RB2 (500 μM) or L-NAME (10 μM). As a positive control the efficacy of the same batch of L-NAME was checked by assessing its effect on electrical field stimulation (EFS)-induced relaxation of pre-contracted rabbit corpus cavernosum according to Thompson *et al.* [24]. The effect of ATP on tissue that was not pre-contracted, and the relative potencies of ADP and UTP vs ATP, were also investigated. Chemicals were all obtained from Sigma Chemical Co. (Poole, Dorset, UK).

In the disease models and their control groups, CSM strips were initially pre-contracted with phenylephrine (100 μM). Cumulative concentration-response curves (CRCs) were then constructed for ATP (1 nM to 10 mM).

The results are expressed as the mean (SEM). Biochemical indices were compared using unpaired Student's *t*-tests. Cumulative response curves were plotted and compared using a two-way ANOVA; in all tests, $P < 0.05$ was considered to indicate significance.

RESULTS

ATP produced concentration-dependent relaxations of pre-contracted CSM strips (Fig. 1a), which were unaffected by L-NAME

FIG. 1. Cumulative CRCs for ATP on phenylephrine pre-contracted CSM. (a) ATP in the absence and presence of L-NAME (10 μM). (b) ATP in the absence and presence of RB2 (500 μM).
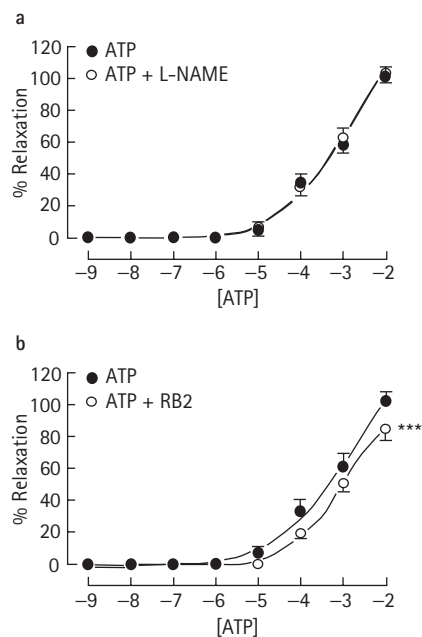
FIG. 2. Cumulative CRCs for P2 receptor agonists on phenylephrine pre-contracted CSM for (a) ATP and ADP, and (b) ATP and UTP.
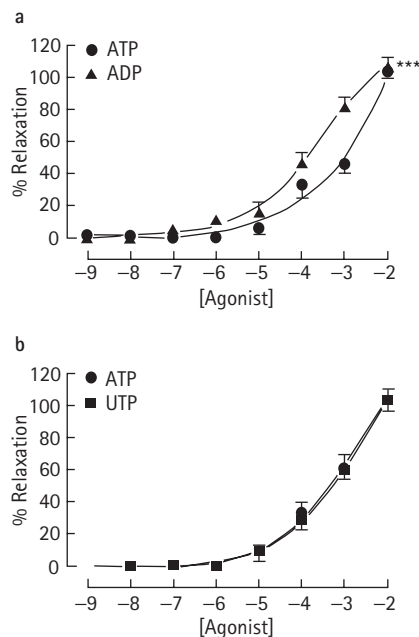
FIG. 3. ATP-mediated CSM relaxation in models of conditions predisposing to ED. (a) Alloxan-induced DM at 6 months. (b) Partial BOO at 6 weeks.
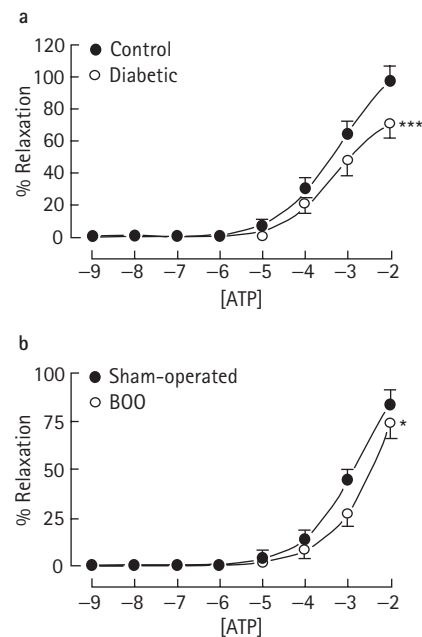
TABLE 1 Body weight and serum glucose concentrations before and after 6 months in rabbits with DM and the controls, and body and bladder weight and serum biochemistry in rabbits with BOO and the controls

Mean (SEM) variable	Control	Diabetic	BOO
DM			
Body weight, kg			
Initial	3.40 (0.02)	3.51 (0.05)	
Final (6 months)	4.70 (0.04)*	3.28 (0.10)†	
Serum glucose, mM			
Initial	7.7 (0.31)	7.3 (0.54)	
Final	7.5 (0.39)	26.3 (1.51)‡	
Final serum [Na$^+$], mM	142 (0.38)	132 (1.0)§	
Final serum [urea], mM	6.34 (0.3)	11.13 (0.6)§	
Final serum [creatinine], mM	93 (2.4)	116 (2.1)§	
BOO			
Body weight, kg			
Initial	3.12 (0.07)		3.09 (0.06)
Final (6 weeks)	3.2 (0.11)		3.15 (0.09)
Bladder weight, g			
Final	3.1 (0.4)		11.8 (2.3)¶
Final serum [Na$^+$], mM	144 (1.6)		142 (2.3)
Final serum [urea], mM	5.8 (0.9)		5.7 (0.6)
Final serum [creatinine], mM	92 (8.3)		92 (5.8)

*Control rabbits gained weight over 6 months (P < 0.001); †DM rabbits lost weight over 6 months (P = 0.04); ‡Final serum [glucose] was higher in DM rabbits than in controls (P < 0.001); §DM rabbits had a lower serum [Na$^+$] and a higher serum [urea] and [creatinine] at 6 months (P < 0.001 for each); ¶Final bladder weight was greater in the BOO group than in the controls (P = 0.002).

(Fig. 1a). RB2 significantly reduced ATP-mediated relaxations (P < 0.001; Fig. 1b), but neither 8-pSPT nor suramin had any effect on ATP responses. ADP also induced concentration-dependent relaxations of pre-contracted CSM strips, which were significantly more potent that ATP (P < 0.001; Fig. 2a); relaxations to UTP were equipotent to ATP (Fig. 2b). ATP had no effect on CSM strips that were not pre-contracted.

There was no significant difference in the initial serum glucose concentrations (non-fasting) in either group of rabbits, but at 6 months it was significantly higher in the DM group. Likewise there was no significant difference in the initial rabbit weights, but after 6 months the DM rabbits had lost weight compared to controls. The DM rabbits had a significantly lower serum Na$^+$ level and higher serum urea and creatinine levels than the control group (Table 1).

CSM strips from the DM and control groups were pre-contracted with phenylephrine; there was no significant difference in tension produced by 100 μM phenylephrine in either group. ATP produced concentration-dependent CSM relaxations in both DM and control tissues (Fig. 3a). The relaxation was

lower in the DM CSM strips than in the control group ($P < 0.001$).

There was no significant difference in body weights from the BOO rabbits and controls. Although all rabbits could void spontaneously, the bladder mass in BOO rabbits was several times that in the controls (Table 1, $P = 0.002$).

Phenylephrine induced similar contractions of CMS strips from the BOO and control rabbits. ATP produced concentration-dependent relaxations of the CSM strips (Fig. 3b), which were lower in the CSM from rabbits with partial BOO than in the controls ($P = 0.039$).

DISCUSSION

The present study confirms that ATP induces rabbit CSM relaxation via P2 receptors, as the P1 receptor antagonist, 8-PT, had no effect on ATP responses. L-NAME had no effect on ATP-mediated CSM relaxations, consistent with earlier reports that, in rabbits and humans, ATP-mediated CSM relaxation is endothelium-independent [8,10]. ATP and UTP were equipotent, suggesting that $P2Y_2$ or $P2Y_4$ receptors were involved, but as adding RB2 reduced the response, it is likely that a $P2Y_4$ receptor mediates the effect [25]. ADP was more potent than ATP, implicating $P2Y_1$ and possibly $P2Y_{12}$ and $P2Y_{13}$ receptors, and making P2X and $P2Y_{2,6}$ and $_{11}$ receptors less likely. Suramin had no effect, confirming that the response to ADP is unlikely to be mediated by P2X receptors or by $P2Y_{2,11}$ or $_{12}$ receptors, which are suramin-sensitive. $P2Y_1$ receptor mRNA was found in the rat corpus cavernosum [13] and the relatively selective agonist $ADP\beta S$ is known to potently relax human CSM [12]. All studies (including the present) have found ATP-mediated relaxation to be endothelium- and NO-independent, while Shalev *et al.* [12] reported that $ADP\beta S$-mediated relaxation was endothelium and NO-dependent. Taken together, this implies that both $P2Y_1$ receptors on the endothelium and $P2Y_4$ receptors on SM are involved.

Corpus cavernosum strips from 24-month-old rabbits are more sensitive to ATP than from 3- and 7-month-old rabbits [17]. Castration (i.e. removal of circulating testosterone) strongly predisposes to ED; both medical castration (using the LHRH analogue, leuprolide) and surgical castration impaired EFS-mediated CSM relaxation and sodium nitroprusside-mediated CSM relaxation (a NO donor) [19]. However, castration had no effect on ATP-mediated CSM relaxation. Chronic steroid abuse predisposes to ED; rabbits, treated for 2 months with testosterone injections had enhanced ADP-mediated cavernosal relaxation, but the ATP-mediated response was unaltered [18].

ED is a common consequence of DM and might affect as many as half of men with DM [26]. In the second part of the present study the serum glucose data in the rabbits injected with alloxan confirmed the successful induction of DM. Previous data from this model confirmed impaired EFS-mediated and sodium nitroprusside-mediated CSM relaxation [27]. The present data showed that ATP-mediated relaxation is also impaired, as well as endothelium-dependent ADP-mediated relaxations. The pre-contractions induced by phenylephrine were no different in the DM and control groups. Downgrading of P2Y receptor expression or alterations in second-messenger pathway signalling function could be responsible.

Much attention has focused on whether prostatectomy for BOO is associated with a greater risk of ED. However, several recent studies indicated that symptomatic patients with untreated BOO are also at greater risk, even when correcting for other risk factors including age [20–22]. Alterations in endothelin receptor distribution in the CSM of rabbits with BOO were reported [23]. The data from the final part of the present study indicates that purinergic signalling might also be implicated in the pathogenesis of ED associated with BOO.

Why abnormalities occur in the corpus cavernosum secondary to bladder pathology is not clear. It is unlikely that the penis sustained physical damage during the process of ligature placing, because this occurs at a distance and was done in both sham and obstructed rabbits; both groups were catheterized during surgery, so catheterization should not account for the changes. Perhaps BOO induces an abnormality in the autonomic nerves which supply both bladder and corpus cavernosum. Alterations in the innervation of the obstructed bladder were reported with an increase in purinergic innervation and decrease in cholinergic innervation in both this model [28] and in the obstructed human bladder [29]. Further work is clearly needed to test this hypothesis.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **CRC**, concentration-response curve; **(C)SM**, (cavernosal) smooth muscle; **RB2**, Reactive Blue 2; **ED**, erectile dysfunction; **DM**, diabetes mellitus; **NO**, nitric oxide; **L–NAME**, L-N^G-nitroarginine methyl ester; **ADPβS**, adenosine-5′-O-(2-thiodiphosphate); **ATPγS**, adenosine 5′-O-(3 thiotriphosphate); **8–pSPT**, 8-p-sulfophenyltheophylline; **EFS**, electrical field stimulation.