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Alterations in purinergic and cholinergic components of contractile responses of isolated detrusor contraction in a rat model of partial bladder outlet obstruction

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OBJECTIVE

To study the effect of 3 weeks of partial bladder outlet obstruction (BOO), compared to a sham operation, on the cholinergic and purinergic components of detrusor contractile responses to agonists and to electrical field stimulation (EFS); the expression of P2X receptor subtypes was also examined.

MATERIALS AND METHODS

Partial BOO was induced in female Sprague-Dawley rats by surgically applying a jeweller's silver 'jump' ring around the urethra, such that the urethra was constricted but not closed. Sham-operated female rats underwent an identical procedure without placement of a ring.

RESULTS

After 3 weeks of partial BOO the rat bladders became significantly hypertrophied, doubling in weight. Spontaneous activity was markedly increased, but the contractile response to a single bolus of KCl (120 mM) was unaltered. The neurogenic-induced contractile responses of strips of detrusor from obstructed bladders were significantly greater than those from sham-operated bladders, and the responses of strips of detrusor from obstructed bladders to EFS showed a significantly greater atropine-sensitive component than sham-operated detrusor. However, the response of detrusor strips to EFS that was susceptible to desensitization by α,β -methylene ATP was not significantly changed in obstructed bladders. The sensitivity of the strips from obstructed bladders to carbachol, ATP and β,γ -methylene ATP was less than in sham-operated detrusor. Immunohistochemical studies showed no difference in the P2X

receptor subtypes expressed on detrusor smooth muscle from obstructed and sham-operated rats.

CONCLUSION

In the rat, after moderate bladder hypertrophy, the atropine-sensitive component was significantly up-regulated, but the ATP-sensitive component was marginally reduced, although not significantly. These results suggest that up-regulation of the P2X component of bladder contraction seen in humans with bladder instability, and in other species models of BOO, is not mirrored in the rat, or occurs later in the pathological process of bladder hypertrophy.

KEYWORDS

bladder, outlet obstruction, P2X, cholinergic, purinergic

INTRODUCTION

Bladder overactivity represents a massive health and economic problem, with symptoms affecting up to 9 million people >40 years old in the UK [1]. The condition of bladder instability is characterized by involuntary bladder contractions causing pressure rises during bladder filling, which result in a strong and uncontrollable urge to pass urine, often leading to incontinence [2]. The mechanisms underlying bladder overactivity are poorly understood. It is clear that there are marked species differences in the proportions of the co-transmitters acetylcholine and ATP in the parasympathetic nerves supplying the bladder of different species including man [3]. In the healthy human bladder, the purinergic component of parasympathetic co-transmission is minor,

although P2 receptors are present on the smooth muscle. However, in pathologically overactive bladders, the purinergic component accounts for up to half of the contraction [4–6]. There was also a significant increase in the purinergic component of detrusor contraction in a rabbit model of partial BOO [7]. However, in a rat model the sensitivity to purinergic receptor agonists was reduced after 10 days of BOO [8] and in a long-term rat obstruction model, only the cholinergic element of electrical field stimulation (EFS)-induced detrusor contractions was increased [9]. Animal models of BOO must be interpreted with caution as they may represent the response to acute retention and subsequent detrusor failure, as indicated by bladder weight increases of several hundred percent and reduced contractile responses to KCl. In the present

study, the effect of partial BOO on the cholinergic and purinergic components of contraction of the rat detrusor were investigated.

MATERIALS AND METHODS

To induce BOO, female Sprague-Dawley rats (173–220 g) were anaesthetized using a mixture of halothane and oxygen. Under sterile conditions, a lower midline incision was made, the peritoneum was mobilized cranially and the prevesical fat was retracted to expose the bladder neck and urethra running on the ventral surface of the vagina. A 1-mm section of proximal urethra was mobilized 2–3 mm from the bladder neck. Care was taken to avoid mobilizing the bladder neck to prevent damage to bladder

neck nerves. Similarly, care was taken to avoid puncturing the large peri-urethral veins. After mobilization of the urethra, a jeweller's silver 'jump' ring with an internal diameter of 1 mm was passed around the urethra and closed exactly. The ring remained loose on the urethra and did not appear to cause immediate obstruction. The bladder was then gently squeezed to ensure it was not fully obstructed and that the urethra had not been punctured. The bladder and wound were irrigated with saline to prevent drying and the formation of adhesions. Usually it was possible to perform the operation without entering the peritoneal cavity. The wound was closed with absorbable suture, and local anaesthetic (0.3 mL 0.25% marcaine), and a single dose of analgesic (0.28 mL temgesic) were administered. The rats were observed overnight in a designated recovery room, and then returned to normal conditions with food and water *ad libitum* the following day. Sham-operated female rats had an identical procedure but with no ring placed. Rats were killed at 3 weeks \pm 2 days by asphyxiation with CO₂ and subsequent cervical dislocation, according to Home Office (UK) regulations covering Schedule 1 procedures.

Bladders were weighed intact after division at the urethra-bladder neck junction. The bladder was divided immediately above the level of the ureters to exclude the bladder neck. The remainder of the bladder was then pinned and divided into strips measuring $\approx 3 \times 20$ mm. Silk ligatures were applied to each end of the detrusor strip; one end was attached to a rigid support and the other to a force displacement transducer (FT03C, Grass Instruments, Quincy, MA, USA). Tissues were mounted in 10 mL organ baths, continually gassed (95% O₂/5% CO₂) and containing Krebs' solution of the following composition (mM): NaCl, 133; KCl, 4.7; NaHCO₃, 16.4; MgSO₄, 0.6; NaH₂PO₄, 1.4; glucose, 7.7; and CaCl₂, 2.5; pH 7.3. Experiments were carried out at 37 \pm 1 °C. Mechanical activity was recorded using the software PowerLab Chart for Windows (Version 4; AD Instruments New South Wales, Australia). Indomethacin (3 μ M) was added to all Krebs' solutions to reduce the spontaneous detrusor activity. An initial load of 1 g was applied to the preparations. Despite allowing the preparations at least 1 h to stabilize, pilot studies found that initial responses to EFS from partially obstructed bladder preparations were inconsistent, and contractions often increased after the first frequency-response curve (FRC); subsequent

FRCs were more consistent. Therefore control contractions were repeated three times to ensure reproducibility. The contractions from the last two FRCs were averaged to give a control contraction.

Strips of detrusor were subjected to EFS (100 V, 0.3 ms, 1–32 Hz, 20 s) every 5 min. Three FRCs were constructed to establish consistent contractile responses. FRCs were subsequently constructed after 20 min in the presence of atropine (1 μ M), or after desensitization with α , β -methylene ATP to establish the relative cholinergic and purinergic components of the contraction. Further FRCs were then constructed in the presence of both antagonists, and then finally in the presence of tetrodotoxin (1 μ M), to determine the part of the response due to direct electrical stimulation of smooth muscle. The Krebs' solution in the organ bath was changed between each set of FRCs.

Non-cumulative concentration-response curves (CRCs) were constructed using carbachol (0.01 μ M to 0.3 mM), ATP (0.1 μ M to 1 mM) and β , γ -methylene ATP (0.1 μ M to 0.3 mM). At the end of the experiment 120 mM KCl was applied. The bladder strips were weighed after completing the experiment.

Responses to EFS were corrected to take into account the tetrodotoxin-insensitive component of the contractions. The contraction to EFS is expressed as the mean (SEM) percentage contraction of the maximum contraction. Contractile responses to non-cumulatively applied agonists are expressed as the mean (SEM) percentage of the maximum response to KCl (120 mM). As the CRC to ATP did not reach a maximum, it was not possible to calculate an EC₅₀ concentration.

Results were assessed using standard statistics, followed by a *post hoc* test to determine if the FRCs were significantly different from each other. Statistical significance was tested by a two-way ANOVA, followed by Bonferroni's test, with $P < 0.05$ considered to indicate statistical significance.

For immunohistochemistry, tissue samples of detrusor were embedded in Tissue-Tek OCT compound (Sakura, Zoederwoude, the Netherlands) and snap-frozen in liquid nitrogen pre-cooled with isopentane. The tissues were sectioned at 12 μ m using a cryostat (Leica CM 3050, Heerbrugg,

Switzerland), thaw-mounted on gelatine-coated slides and air-dried at room temperature. The slides were stored at -20 °C and allowed to return to room temperature for at least 10 min before use.

The immunogens used were peptides corresponding to 15 receptor type specific amino acids in the C-terminal region of the P2X₁₋₇ receptor subtypes (Roche Palo Alto, CA, USA). The avidin-biotin technique was used, as described by Llewellyn-Smith *et al.* [10,11]. Briefly, the slides were fixed in 4% formaldehyde and 0.2% of a saturated picric acid solution in 0.1 M phosphate buffer for 2 min. To inactivate endogenous peroxidase, the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Non-specific binding was blocked by incubating with 10% normal horse serum in PBS containing 0.05% thimerosal (Merthiolate) for 20 min. The P2X antibodies were diluted to 5 μ g/mL (determined by prior titration) with normal horse serum and the sections incubated with primary antibodies overnight at room temperature. The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) used at 1 : 500 for 1 h. Sections underwent a further incubation with extravidin peroxidase (Sigma Chemical Co., Poole, UK) at 1 : 1000 for 1 h. The reaction product was visualized using the nickel-diaminobenzidine enhancement technique or using streptavidin fluorescein isothiocyanate immunofluorescence. The specimens were dehydrated in xylene and mounted in Eukitt (VWR International, Poole, UK). Controls were incubated with pre-immune IgG and antibodies pre-absorbed with the homologous peptides and omission of the primary antibody; there was minimal staining under such conditions. The results were documented using a high-definition light microscope (R400, Edge Scientific Instruments, Santa Monica, CA, USA). Pictures were stored using digital camera technology (Leica 2000, Leica, Heerbrugg, Switzerland).

RESULTS

Partial BOO did not adversely affect rat growth, as assessed over 3 weeks; the mean body weight increase was 22.5 (3.7) g in sham-operated rats and 27.6 (3.9) g in rats with partial BOO ($P = 0.4$). Two rats died ≈ 3 days after surgery from urinary retention. In both cases the ring was closed so that it

overlapped very slightly. In all other cases the ring was closed exactly. This was important, as even the smallest gap resulted in failure to induce bladder hypertrophy. There were no other postoperative complications and the procedure appeared to be very well tolerated. It was not necessary to massage bladders immediately after surgery to facilitate voiding, as in other studies [9].

The weight of sham-operated rat bladders was 70.3 (1.3) mg; the partially obstructed bladders were about twice as heavy, at 141.8 (14.5) mg (unpaired *t*-test, $P = 0.0016$; Fig. 1a). The detrusor strip weight was 11.1 (0.7) mg from sham-operated rats and 16.6 (0.8) mg from partial BOO rats.

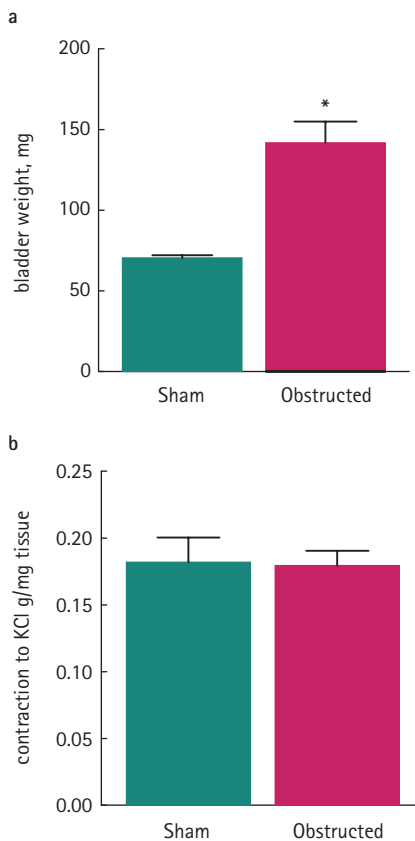
There was no significant difference in the response of detrusor strips to 120 mM KCl between sham-operated rats and rats with partial BOO, at 0.182 (0.019) g per g of tissue and 0.179 (0.011) g per g of tissue, respectively (Fig. 1b).

EFS induced tetrodotoxin-sensitive frequency-dependent contractions. There were small tetrodotoxin-insensitive contractions at the higher frequencies, consistent with direct muscle stimulation; all results are adjusted for this. The contractile responses of partial BOO detrusor to EFS were significantly greater than in sham-operated detrusor. The mean peak contraction at 16 Hz was 80% greater in partial BOO detrusor, at 111.3 (12.8)% and 62.5 (11.7)% of the contraction for the KCl response, respectively (two-way ANOVA, $P = 0.0015$; Fig. 2).

The proportion of the control contraction that was inhibited by atropine (1 μM) was greater for partial BOO detrusor at all frequencies tested and increased with increasing frequency. Maximum inhibition occurred at 16 Hz and was a mean (range) 81 (42–81)% reduction in contraction. The percentage of initial contraction inhibited by atropine (1 μM) in sham-operated rats also increased with increasing frequency, but was less, and at 16 Hz was 47 (24–56)% (two-way ANOVA, $P = 0.0013$; Fig. 2).

The proportion of the control detrusor contraction that was inhibited after desensitization with α, β -methylene ATP (2 \times 100 μM) was greater in sham-operated rats than in partial BOO rats. This component was constant in sham-operated rats, at a mean inhibition of 73% (Fig. 3a). In partial

FIG. 1. (a) Mean (SEM) bladder weights (mg) from five sham-operated controls and six rats with partial BOO at 3 weeks. (b) Comparison of tension generated by 120 mM KCl on detrusor strips from sham-operated controls (24 strips, five bladders) and rats with partial BOO after 3 weeks (31 strips, six bladders).

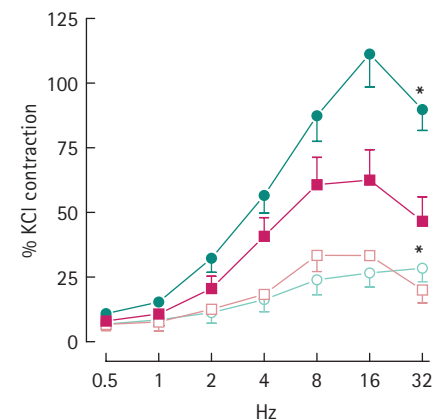


BOO rats, the α, β -methylene ATP-induced inhibition was more variable and inconsistently increased with frequency. At 0.5 Hz inhibition was 50% and at 32 Hz was 70%, with a mean inhibition of 62% (Fig. 3b). The difference in the purinergic components was not significant (two-way ANOVA, $P = 0.16$).

The combination of atropine (1 μM) and α, β -methylene ATP (2 \times 100 μM) caused significantly greater inhibition in partial BOO rats; there was virtually no contraction under these circumstances (Fig. 3b), but in sham-operated rats there was some residual contraction at the higher frequencies tested (Fig. 3a).

Detrusor strips contracted in a concentration-dependent manner to carbachol (0.01 μM to 0.3 mM). The sensitivity of detrusor strips from partial BOO rats to carbachol was

FIG. 2. The FRC showing contraction to EFS (100 V, 0.3 ms, 0.5–32 Hz, 20 s) of sham-operated control (five; red closed square) and partial BOO (six; green closed circle) strips of detrusor, and subsequently, in the presence of atropine (1 μM), sham-operated (light red open square), partial BOO (light green open circle). All symbols show the mean (SEM) percentage contraction of the response to 120 mM KCl (unless masked by symbol). All data shown were adjusted to subtract the tetrodotoxin-insensitive component. Asterisk indicates significance ($P \leq 0.05$).

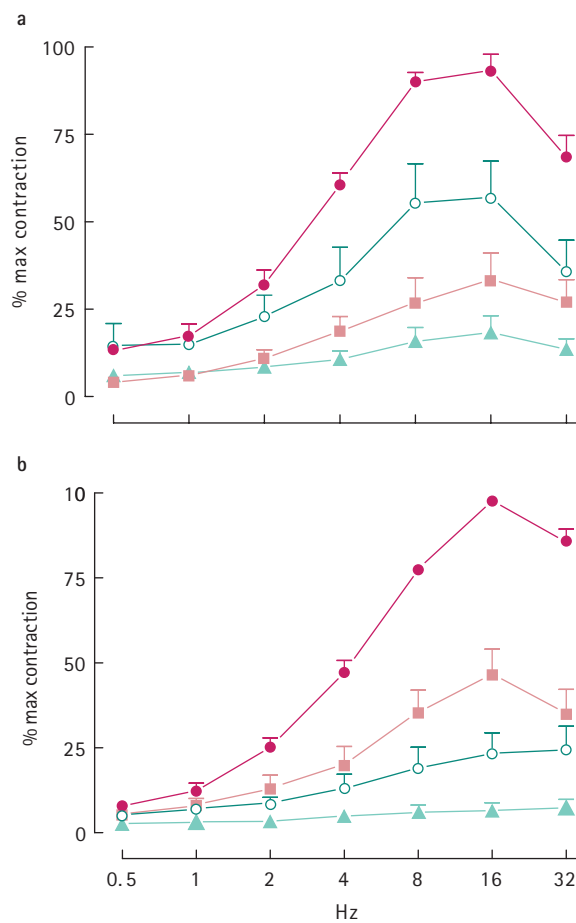


significantly less than that with sham-operated rats. The EC_{50} was 0.96 μM for sham-operated rats and 3.0 μM for partial BOO rats (two-way ANOVA, $P = 0.001$; Fig. 4a).

Detrusor strips contracted in a concentration-dependent manner in response to both ATP (0.1 μM to 1 mM) and β, γ -methylene ATP (0.1 μM to 0.3 mM). The sensitivity to the more stable purinergic agonist β, γ -methylene ATP was significantly less in the partial BOO rats (two-way ANOVA, $P = 0.0078$; Fig. 4b). The responses of detrusor strips to ATP were smaller than those for β, γ -methylene ATP. The sensitivity to ATP was also significantly less in the partial BOO rats (two-way ANOVA, $P = 0.0031$; Fig. 5).

There was P2X_1 receptor expression on the detrusor smooth muscle membrane from both sham-operated and partial BOO rats, with no discernible difference in the expression (Fig. 6). There was minimal expression of P2X_2 receptor within smooth muscle cells from either group, there was P2X_3 receptor expression in a subepithelial position in both groups, but there was no smooth muscle expression. There was some expression of P2X_4 receptors on the transitional epithelial cells from both groups, no expression of P2X_5 receptors, but some

FIG. 3. FRCs showing initial contraction to EFS (100 V, 0.3 ms, 0.5–32 Hz, 20 s) of either sham-operated (a) or partial BOO (b) strips of detrusor (red closed circle). Subsequently, after desensitization with α,β -methylene ATP (light red closed square) (five sham and six partial BOO) in the presence of atropine (1 μM) (green open circle) or both atropine (1 μM) and after desensitization with α,β -methylene ATP (light green closed triangle). All symbols show the mean (SEM) percentage maximum contraction, unless masked by the symbol. All data shown were adjusted to subtract the tetrodotoxin-insensitive component.



expression of P2X₆ receptors on the basal membrane from both groups. There was no expression of P2X₇ receptors.

DISCUSSION

The rat model of partial BOO has been shown to cause detrusor instability [12,13]; in some studies, very large bladder mass increases and reduced KCl responses were reported [9]. These studies must be interpreted with caution, as they may be more representative of the recovery response of the bladder to acute obstruction. The present partial BOO model was well tolerated and resulted in a relatively mild increase in bladder mass, with evidence of genuine detrusor hypertrophy, increased nerve-mediated contraction and marked spontaneous activity. As a consequence of the hypertrophy the strips from the obstructed rats were slightly heavier but small differences in detrusor strip size have not been shown to significantly affect

tension responses [14]. Macroscopically, the bladder detrusor smooth muscle and epithelium were thickened; hypertrophy was confirmed histochemically. There was no deterioration in smooth muscle contractility as assessed by the contractile response to KCl. In the present study the cholinergic component of the nerve-induced contraction accounted for up to 80% in partially obstructed bladders, although only 50% in sham-operated bladders. The purinergic component was reduced, although not significantly. In a longer (12 week) BOO model in the rat there was a significant increase in the cholinergic component and no significant increase in the purinergic component, but the contraction to KCl was significantly reduced, indicating reduced bladder contractility [9].

Purinergic co-transmission in the contraction of the bladder smooth muscle of many animals is well documented [3]. In the normal human bladder it is thought to have a minimal contribution to normal physiological contraction [5]. However, P2X receptors are

expressed on normal human detrusor smooth muscle, and there was contractile function with exogenous ATP [6]. Human studies are challenging due to difficulty in confirming bladder overactivity, and ensuring that controls represent 'normal' tissue. Several studies have suggested that the purinergic component of human bladder contraction is substantially up-regulated in the pathological state. The purinergic co-transmission component is increased to account for 40% of the contraction in interstitial cystitis [4], up to 50% in idiopathic female detrusor instability [6], and in cases of proven bladder instability there is significant up-regulation [5]. It has been reported that, with age, the purinergic component of human bladder contraction is increased and the cholinergic component reduced [15]. Another study contradicts this, suggesting that the purinergic component, as assessed by antagonism, is only 5% in overactive detrusors and 3% in controls, with a significant up-regulation of the myogenic or tetrodotoxin-resistant component in overactive bladder tissue [16]. The up-regulation of the purinergic component cannot be explained by altered sensitivity, as detrusor myocytes from stable and unstable bladders were equally sensitive to cholinergic and purinergic agonists [17]. A reduction in ecto-ATPase enzyme activity was reported in unstable or obstructed human bladders and this may account for the increased potency of ATP in these pathological states [18]. There is greater expression of the P2X₂ receptor in detrusor instability [6].

The increase in the cholinergic component of detrusor contractility in the present study cannot be explained by denervation supersensitivity, as although the nerve-stimulated response was increased the response to exogenous carbachol was reduced. Brading [19] suggested that a feature of bladder overactivity is denervation supersensitivity, in which there is a reduction in the number of excitatory nerves and a reduction in acetylcholinesterase, resulting in agonist supersensitivity. By contrast, it was suggested that there is an increase in cholinergic nerve proliferation in BOO [20,21]. It was proposed that the relative ratio of parasympathetic co-transmitters could change in pathological conditions [3]. A study into the neuroanatomical changes of hypertrophied rat detrusor showed a modest increase in P-glycoprotein-staining nerves around smooth muscle bundles, but minimal change within the smooth muscle

bundles. By contrast, nerves expressing acetylcholinesterase within the bundles were reduced, although between bundles they were unchanged [22]. In another rat study of mild obstruction there was no change in choline acetyltransferase expression, and no dysfunction in responses to EFS [23]. In the present study, the neurogenic atropine-sensitive component was increased, consistent with a reduction in acetylcholinesterase. However, it does not explain the reduced sensitivity to carbachol, although the carbachol sensitivity would be independent of acetylcholinesterase because it is not hydrolysable. The sensitivity to all agonists tested was reduced, suggesting a common change in the contractile mechanism. In previous rat models, the detrusor rapidly adapted to partial BOO, with bladder weight increasing up to 12-fold in 6 weeks, with the fastest growth rate at 3 days after obstruction [22,24]. The growth is mainly accounted for by smooth muscle hypertrophy, with the smooth muscle cross-section increasing significantly. The collagen content also increased, but the relative concentration was reduced to a third of control levels. The smooth muscle bundles were preserved, with the collagen increase being mostly between bundles, with minimal collagen infiltration within bundles [25,26]. It therefore seems unlikely that the reduced sensitivity to all exogenous agonists in the present study could be explained by tissue fibrosis obstructing agonist permeability. The response to KCl was similar, suggesting no fundamental change in smooth muscle contractility. The present results therefore suggest a change in the expression of receptors or transduction mechanisms.

The rat bladder expresses several P2X receptor subtypes, with strong expression of P2X₁ receptors on detrusor smooth muscle [27]. In the present study, there was no difference between normal and obstructed bladder in the expression of P2X receptor subtypes. Subtype expression concurred with previous reports [27]. In conscious rats, bladder contraction was induced by ATP, and then reduced after pretreatment with α,β -methylene ATP [28]. Similarly in the P2X₁ receptor-deficient mouse, despite expression of other subtypes of P2X receptors, no detrusor contractions were recorded to the P2X agonists ATP, α,β -methylene ATP, and β,γ -methylene ATP. This was supported by patch-clamp studies on detrusor smooth muscle, showing no effect on the holding

FIG. 4. CRCs for (a) carbachol and (b) for β,γ -methylene ATP in sham-operated controls (five; red closed square) or partial BOO (six; green closed circle). All symbols show the mean (SEM) percentage of the contraction to KCl (120 mM) unless masked by the symbol. Asterisk indicates significance ($P \leq 0.05$).

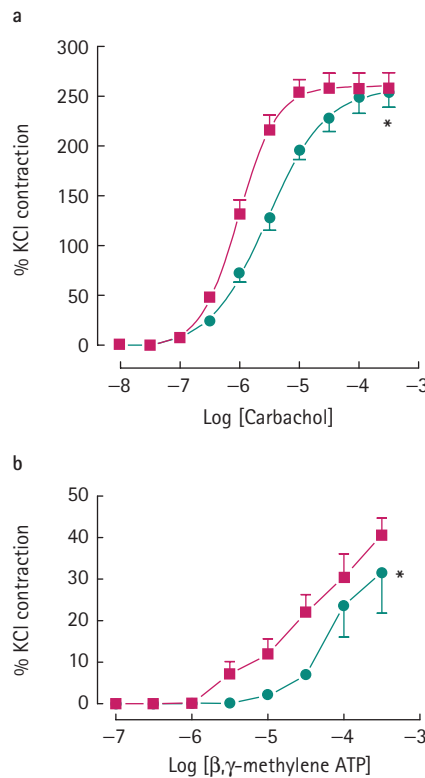


FIG. 5. The CRC for ATP in sham-operated controls (five; red closed square) or partial BOO (six; green closed circle). All symbols show the mean (SEM) percentage of the contraction to KCl (120 mM), unless masked by the symbol. Asterisk indicates significance ($P \leq 0.05$).

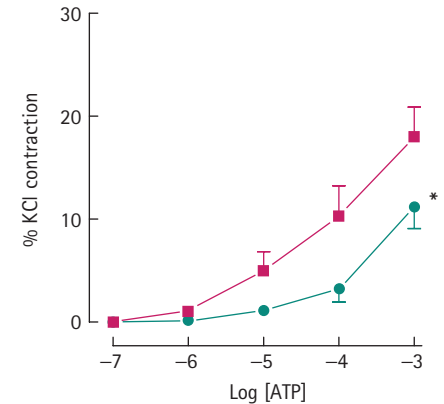
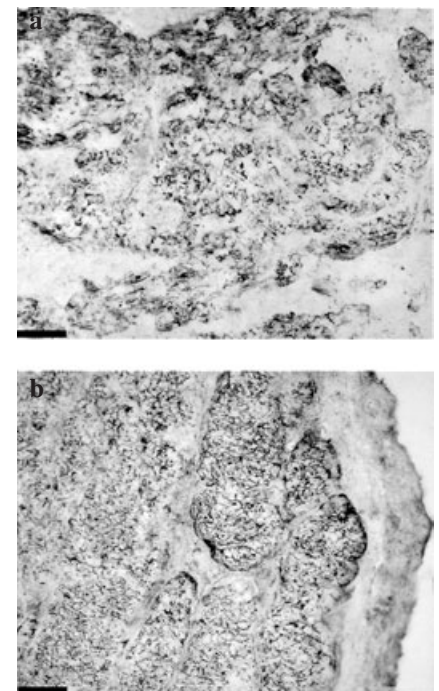


FIG. 6. A photomicrograph showing P2X₁ receptor immunostaining expression on the membrane of detrusor smooth muscle of: a, sham-operated control rats (there was no epithelial expression); and b, of partial BOO rats; there was no epithelial expression. All scale bars are 50 μ m.



current in P2X₁ receptor-deficient mice. The bladders of P2X₁ receptor-deficient mice were functionally and morphologically normal. It was thought that the cholinergic element of the bladder contraction was sufficient to maintain function [29]. This is in contrast to contraction of the vas deferens from P2X₁ receptor-deficient mice, which was markedly reduced, resulting in infertility [30]. The evidence strongly suggests that the functional P2X receptor involved in smooth muscle contraction in rat, mouse and probably human bladder is the P2X₁ subtype. In the rabbit testis where α,β -methylene ATP is less potent than ATP, the dominant P2X receptor subtype may be P2X₂ [31]. The function of other P2X subtypes expressed in smooth muscle is less clear, as is the functional role of the suggested heteromultimers P2X_{1/5} and P2X_{1/2}. The P2X₃ receptor has a mechano-sensory role in micturition in mice, and P2X₃ receptor-null mice have bladder hyporeflexia [32,33]. There was no discernible reduction in the

expression of P2X receptors. It is therefore possible that the neurogenic purinergic component is marginally reduced because of either reduced ATP release or increased ecto-ATPase activity.

This study shows that the cholinergic component of rat detrusor contraction is enhanced in mild obstruction and the purinergic component is marginally reduced. This is in contrast to the rabbit and human, in which the reverse occurs. We suggest that the rat detrusor adapts to obstruction in a different manner to that of man, or that purinergic up-regulation is not a feature of early adaptation.

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CONFLICT OF INTEREST

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Abbreviations: FRC, frequency response curve; CRC, concentration response curve; EFS, electrical field stimulation.