The Use of the Isolated Mouse Whole Bladder for Investigating Bladder Overactivity

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Running Title: Functional assay of isolated mouse whole bladder overactivity

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Text pages 16
References 40
Figures 10
Number of words in abstract 199
Number of words in introduction 436
Number of words in discussion 1,487

Abbreviations: Carbachol CCh, Bradykinin BK, Pirenzepine PRZPN, Pulse Width PW, Train Duration TD, TTX Tetrodotoxin.
ABSTRACT

The isolated mouse whole bladder was used to study in vitro bladder overactivity evoked by intramural nerve sensitization with bradykinin, mimicking neurogenic bladder overactivity secondary to bladder inflammation. Intravesical pressure responses to intramural electrical stimulation (EFS) of intramural nerves were measured under isovolumetric condition. Validation showed that carbachol produced a dose response curve closely mirroring that observed in the isolated muscle strips, and demonstrated the dual nature of electrically evoked neurotransmission, consisting of a cholinergic component largely mediated by M3 receptors and a purinergic component mediated by P2X receptors. ATP generated a biphasic dose response curve, suggesting that the P2X receptors may be heterogeneous in distribution. Characterization of bradykinin receptors showed bradykinin to be extremely potent in exciting the bladder, producing a dose response curve with an EC50 of 90nM, and bradykinin also enhanced electrically evoked bladder contractions. These effects were inhibited by the B2 receptor antagonist HOE 140 but not the B1 receptor antagonist desArg10HOE 140 and were also modulated by α,β-methyleneATP. The isolated mouse whole bladder has proved a viable, robust model in which to demonstrate the pharmacological characteristic of the bladder, and adds to the repertoire of in vitro tools for investigating potential therapeutic agents.
INTRODUCTION

The mammalian bladder is an integral part of the lower urinary tract with two important mutually exclusive functions, the storage of urine (continence) and periodic elimination of urine (acting as a pump) i.e. micturition. These functions depend on the intricate network of detrusor smooth muscle and its extensive innervation from prevertebral and intramural ganglia (Daniel et al., 1983). The muscle bundles are not arranged in a definite pattern but form an interlacing network in which bundles freely criss-crosses one another (Dixon and Gosling, 1987). It is this unique characteristic arrangement that underlies the mechanics of bladder function conferring the detrusor smooth muscle the ability to simultaneously contract in a multidimensional fashion allowing the bladder transition between detrusor stretching (isovolumetric) that occurs during continence, and contraction required for urine expulsion (Damaser, 1999).

Clinically, bladder function is evaluated through biomechanical characteristics such as pressure, volume and urine flow rate (Damaser, 1999). For any in vitro model to be clinically relevant it is imperative that these factors be taken into consideration during model development. Much of the knowledge of urinary bladder function comes from in vitro muscle strips studies, which have a functional utility limited by the fact that strip contraction can only be recorded in one plane: results do not reflect the potentially complex interactions that underlie the pressure-volume relationship of the intact bladder (Levin et al., 1983; Levin et al., 2000). The isolated mouse whole bladder, whilst technically challenging offers more physiological relevance, since it contains all the elements including intramural ganglia, and measurement of function is possible in terms of volume-pressure relationships. Additionally, the availability of transgenic and gene-knockout animals (Bishop, 1999) makes a mouse model very attractive for biomedical research.
In this study we have employed the whole bladder to investigate the underlying cause of overactive bladder secondary to chronic inflammatory conditions such as hemorrhagic cystitis or interstitial cystitis. One of the contributing factors to these conditions is a functional change in the primary afferents brought about by the sensitization of both mechanosensitive and C-fibres afferents by potent inflammatory mediators such as bradykinin (Marceau et al., 1980; Maggi et al., 1989).

Evidence has linked the activity of bradykinin to efferent purinergic neurotransmission in rat isolated muscle strips (Acevedo et al., 1990; Patra and Westfall, 1996). The goals of this study are to validate the isolated mouse bladder model, to elicit neuronal hyperactivity with bradykinin (as the noxious stimulus) and to shed light on any functional relationship between bradykinin and P2X receptor subtypes in this preparation.

An account of the findings has been presented previously to Urological Research Society annual meeting (Fabiyi and Brading, 2005).
MATERIALS AND METHODS

All animal handling and procedures were conducted in accordance to the United Kingdom Home Office regulations of Animals (Scientific Procedures) Act 1986. Female CD1 mice (25g) were killed by inhalation of rising concentration of CO₂, followed by cervical dislocation. Whole bladders were removed and transferred into Krebs’ solution (mmol/l: NaCl 120, KCl 5.9 NaHCO₃ 15.4, CaCl₂ 2.5, MgCl₂ 1.2, glucose 11.5 equilibrated with 97% O₂ and 3% CO₂) where they were stored in the refrigerator (at 4°C) until processing.

Muscle strips were prepared with the aid of dissecting microscope, the bladder was pinned down at the apex of the dome, connective tissue was then removed and the bladder split open along the ventral surface to expose the urothelium which was then carefully peeled off from the muscle layer and discarded. The tissue was cut longitudinally into strips of approximately 5mm by 1mm. Each strip was then mounted vertically in a 0.2 ml capacity, superfusion organ bath between two recessed platinum electrodes through which electrical field stimulation (EFS) was delivered (see Brading and Sibley, 1982 for description of apparatus). The strips were constantly superfused with Krebs’ solution (37 °C) at a rate of 1 ml / min. A stretch equivalent to 1 gm force was applied and tissues equilibrated for at least 60 minutes before starting a procedure. Drugs were delivered by substituting drug containing solutions of the desired concentration for Krebs’ solutions. Electrical field stimulation was delivered by Grass S48 stimulator at parameters of 0.05ms pulse width (PW), 3s train duration (TD), 50V at varying frequency. Isometric tension generated by the strips was measured using isometric force displacement transducers, Pioden Dynamometer UFI transducers (Pioden Controls Ltd, Canterbury, Kent) or AD Instruments MLT050/D force transducers, amplified (Harvard Transducer/Amplifier, Harvard Apparatus Ltd, Edenbridge, Kent) and analyzed using MacLab Data Acquisition system (AD Instruments,
Dose response curves for carbachol (CCh, 0.1µM-0.3mM) were constructed by subjecting the preparations to a sequence of successive twenty seconds applications of increasing doses of drugs with intervening washout periods of at least twenty minutes. Frequency response curves were constructed in the absence and presence of either atropine (1µM) or a cocktail of atropine and α,β,methyleneATP (10µM). Responses were also obtained in the presence of tetrodotoxin (TTX) to ensure that contractions were solely due to selective stimulation of intramural nerves and not direct smooth muscle stimulation.

For the isolated whole bladder experiments, the ureters were ligated and the bladder was excised at the distal urethral level. Any superfluous tissues were removed around the urethra and bladder neck. Using a silk ligature the proximal end of the urethra was tied around a stainless steel pipe (4-5mm long, 0.25mm, internal diameter) that was inserted into P-10 (0.28mm internal diameter) tubing to provide a water-tight seal and prevent leakage from the bladder once filled. The pipe was sealed to the base of stainless steel J-tube (55mm long, 0.58mm in diameter) which was clamped in place. Through the J tube the bladder was suspended in a 20 ml jacketed organ bath containing oxygenated Krebs’ solution at 37° C, and connected to a syringe pump (Harvard Apparatus Ltd, Edenbridge, UK) and pressure transducer for measurement of intravesical pressure. Data were acquired and analyzed using MacLab Data Acquisition system (AD Instruments, Sydney, Australia) or AD Instruments OCTAL Bridge Amplifier connected to Power Lab/8SP (AD Instruments, Sydney, Australia) and Chart v3.6 software (Macintosh) or Chart v5 (PC).
Bladders were slowly filled with saline (0.1-0.15ml) at the rate of 0.8ml/hr, and allowed to equilibrate for an hour. Prior to commencement of experiments bladders were tested for viability by exposing them to 3µM carbachol, and as soon as the maximum response was reached, washed for five minutes by overflow to prevent desensitization. Electrical field stimulation (EFS) was applied through platinum plates, one on each side of the bladder. Stimulus parameters were: pulse width 0.2ms (PW), train duration (TD) 3s, 30 or 35s, frequency 20Hz and voltage 50V. Intravesical pressure was measured, which reflects contraction of the detrusor. The bladder was exposed to drugs by exogenously applying the drug into the organ bath, and intravesically as required. The duration of exposure of bladder to the drug depended on the protocol of the experiment as described in the legends.

Experiments were run in normal Krebs’ solution with a maximum volume in the organ bath not exceeding 17ml. Drugs were applied exogenously in the organ bath with a test volume not exceeding 0.5ml to achieve the final concentration. Tetrodotoxin citrate from Alomone Laboratories, Jerusalem, Israel was employed to establish the stimulation parameters at which intramural nerves were selectively stimulated. The cholinergic component was characterized by using a stable analogue of acetylcholine, carbachol (CCh), a non selective muscarinic receptor antagonist, atropine and a range of muscarinic selective antagonists including the M₁ selective antagonist pirenzepine (5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6H pyrido[2,3-b] [1,4] benzodiacepin-6-one dihydrochloride), the M₂ selective antagonist AF-DX116 (11-2[(diethylamino)methyl]-1-piperidinyl)-acetyl]-5,11-dihydro-6Hpyrido(2,3b)(1,4)benzdiazepine-one), the M₃ selective antagonist 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methobromide) and the M₄ selective antagonist PD 102807 ((3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-12H-isoquino[1,2-b]pyrrolo[3,2f][1,3]benzoxazine-1-carboxylic acid ethyl ester);p-F-
HHSiD, para fluoro hexahydrosiladifenidol). The purinergic component was characterized with the aid of purinergic agonists which includes ATP & the stable analogue of ATP, $\alpha,\beta$-methyleneATP which rapidly desensitized the receptors. For the characterization of bradykinin receptors, the antagonists used were desArg^{10} HOE 140 (H-D-Arf-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-OH) and HOE 140 (D-Arg^{0}-Arg^{1}-Pro^{2}-Hyp^{3}-Gly^{4}-Thi^{5}-Ser^{6}-D-Tic^{7}-Oic^{8}-Arg^{9}) which are selective B_{1} and B_{2} antagonist respectively. Stock solutions were prepared in either distilled water or dimethyl sulfoxide (DMSO) and stored in aliquots at -30°C. All the above reagents were purchased from Sigma Chemical Co. (Poole, UK).
RESULTS

Validation of the whole bladder model

To establish the optimum volume for the isolated bladder, volume-pressure response curves were constructed (fig. 1). The maximum pressure response to EFS was observed at 0.18ml with an intravesical pressure of 23.6 ± 5.0 cmH20 (99.5% response), but below this, near maximum responses occurred at bladder volumes of 0.1 ml. On the basis of these observations subsequent studies were carried out over the volume range 0.12-0.15ml. These test volumes have been further validated by a recent publication of Langou (2006) in which they found in a similar model, that the peak contraction occurred at a volume range of between 0.1 and 0.12ml. EFS parameters sensitive to TTX were determined as indicated in fig. 2. From these results parameters of 0.2ms PW, 3s TD, 50V and 20Hz were used as standard EFS parameters in the subsequent studies. Responses were sensitive to TTX (0.3µM).

To establish whether the dual nature of neurotransmission seen in other mammalian bladders also applied to the mouse, the sequential effects of atropine and atropine plus α,β,methyleneATP on the frequency response curves were determined, and the results compared with similar experiments on isolated muscle strips, as shown in Figure 3. In both preparations, atropine (1µM) inhibited the response by about 50%, the additional presence of α,β,methyleneATP (10µM) greatly reduced the responses in the whole bladder and almost completely abolished them in the isolated strips. The responses produced in the presence of atropine and α,β,methyleneATP appear not to differ from the response produced in the presence of TTX alone (fig. 3). Further analysis shows that atropine insignificantly reduced responses at lower frequencies (1-5Hz) but produced substantial reduction at frequency above 20Hz with maximum intravesical pressure reduction of 44% (fig. 3a). In contrast, the desensitization of purinoceptors...
by α,β-methyleneATP markedly reduced responses over all the frequency range tested with approximately 75% reduction in response observed at both 1Hz and 20Hz (fig. 3a).

Further validation studies compared non-cumulative carbachol dose response curves in the whole bladder preparation with the muscle strips preparations. Carbachol produced a characteristic sigmoid dose-dependent increase in tonic bladder contraction in both models yielding an EC50 of approximately 2.4µM and 3.6µM respectively. Consequently 3µM carbachol was used as a standard concentration for subsequent studies in the isolated mouse whole bladder.

Differential effects of selective muscarinic receptor antagonists on mouse whole bladder

The purpose of these experiments was to characterize the muscarinic receptor subunits mediating direct bladder contraction and neuronally-mediated bladder contraction, by evaluating the effect of a range of selective antagonists (pirenzepine, AF-DX116, 4-DAMP and PD 102807) on bladder contraction to 3µM carbachol, and to electrical field stimulation (Fig 4). The doses of pirenzepine (0.3µM) and 4-DAMP (10nM) tested were determined from the study of Giglio (2001) on guinea-pig urinary bladder strips. The dose for AF-DX116 (1µM) was based on the work of Lagou (2006). In the present study, exogenous application of carbachol elicited a robust contractile response of 29.7 ± 2.8cmH2O. As expected the vehicle (DMSO), had no effect (0.58 ± 5.7%), 4-DAMP (10nM), AF-DX116 (1µM) and pirenzepine (0.3µM), all significantly reduced the response by 75.7 ± 2.8, 51.09 ± 6.3 and 43.9 ± 3.8% respectively (fig. 4). PD 102807 (1µM) did not significantly affect the action of carbachol (6.2 ± 3.6%). Similar effects were seen on the contractile response to EFS (control: 21.6 ± 3.0 cmH2O). 4-DAMP (10nM), pirenzepine (0.3µM),
AF-DX116 (1µM) all caused a significant reduction of 41.3 ± 6.7, 25.4 ± 11.2 and 21.5 ± 7.8% respectively. PD102807 (1µM) had no significant effect (fig. 4).

Effects of ATP on mouse whole bladder

The whole bladder also contracted to applied ATP, but the maximum response was not reached even at a concentration of 3mM, and the curve appeared biphasic. Further characterization of purinergic receptors was conducted as indicated in fig. 5. α,β,methyleneATP-induced desensitization of P2X receptors was evoked by three consecutive additions of α,β,methyleneATP at 0.01mM, 0.1mM and 0.1mM (fig. 5a). The first application generated a transient increase in intravesical pressure reaching 25.1 ± 3.4cmH2O, the second transiently increased pressure to 10.40 ± 3.40cmH2O while the third application only evoked a response of 1.3 ± 0.35cmH2O confirming almost complete desensitization of P2X receptors. Exogenous application of ATP (3mM) before and after purinoceptors desensitization produced intravesical pressure of 15.8 ± 1.7 and 6.2 ± 1.0cmH2O respectively, suggesting that desensitization only partially inhibited the response to ATP (by approximately 60%, fig. 5b).

Effects of bradykinin on mouse whole bladder

Bath application of bradykinin produced a slowly developing tonic contraction with a maximum effect averaging 78.1 ± 7.3% of that to 3µM carbachol yielding an extremely potent EC50 of 90nM (fig. 6a). Intravesical application of 1µM bradykinin produced an enhancement of the EFS frequency-response curve. At 20Hz, a response of 82.0 ± 6.7% of that to 3µM carbachol, was obtained, compared to a response of 63.3 ± 5.8% without bradykinin. This effect was completely abolished by TTX (0.3µM) demonstrating the neuronal origin of the contraction (fig. 6b). Bath application of bradykinin (10nM) also transiently enhanced the response to transmural electrical
stimulation. The enhancement was greatest (44.8 ± 7.3%) at five minutes post bradykinin application; thereafter it progressively returned to the control levels over the next 30 minutes (fig. 7a/b).

HOE 140, a selective B2 antagonist almost completely suppressed the bradykinin-induced bladder contraction and significantly reduced (to -4.4 ± 6.9% of control) the enhancement of the response to EFS. On the other hand desArg10HOE 140 displayed no significant activity on either bradykinin-induced bladder contraction or bradykinin-induced potentiation of EFS mediated bladder contraction (fig. 8).

Interaction between receptors
Experiments to examine the influence of bradykinin on either ATP or CCh-induced bladder contractions showed that bradykinin did not significantly potentiate the effect of ATP (fig. 9a) nor did it appreciably affect carbachol-induced contraction (fig. 9b).

These next set of experiments investigated whether atropine or α,β,methyleneATP can modulate the effect of bradykinin. Treatment with atropine significantly reduced the response to EFS, without affecting either the direct contractile effect of bradykinin on the bladder or the potentiation of the EFS response (fig. 8/10a). In contrast, although treatment with α,β,methyleneATP also reduced the bladder response to EFS, it also significantly enhanced the bradykinin induced potentiation of EFS without affecting direct contractile effect of bradykinin (figs 8/10b).
DISCUSSION

The present findings confirm that the mouse bladder response to transmural electrical stimulation consists of two components: cholinergic, mediated largely through M3 receptors and purinergic, mediated through P2X purinoceptors, as seen in the rat and guinea-pig (Burnstock et al., 1978; Brading and William, 1990).

Validation experiments showed that external application of carbachol induced a dose response curve that compares very well with that produced in isolated muscle strips. Five subtypes of muscarinic receptor have been identified molecularly and pharmacologically up to date and are denoted as M1 through M5 (Caulfield and Birdsall, 1998). In the human bladder, mRNA for all the receptor subtypes has been found (Sigala et al., 2002) however only two of these subtypes, the M2 and M3 are considered to have any physiological significance in micturition.

The selective muscarinic antagonists apart from the M4 selective antagonist PD 102807, tested at their optimum concentrations, reduced the contractile effects of CCh and EFS to a certain degree. However the M3 selective antagonist 4-DAMP appeared to be the most effective. This finding supports the notion that M3 receptors are the main receptor subtype responsible for both direct (Hedge et al., 1997; Chess-Williams, 2002) and indirect (Chopping and Elgen 2001a) bladder contractions. It has been proposed that the mechanism of action of the M2 receptor subunits is indirect, through inhibition of β-adrenoceptors-mediated relaxation to facilitate micturition (Hedge et al., 1997), and that the M1 receptor subtype facilitates the release of ACh in response to prolonged high frequency nerve firing associated with voiding (Somogyi et al., 1994). While although the results of the current study are consistent with a pre-junctional activity of these receptors by the virtue of their ability to modulate neuronally mediated bladder contractions, it
also supports some post-junctional activity since the antagonists appears to be more effective in antagonizing the contractile effect of CCh than EFS (Hedge et al., 1997). Our study also clearly shows that the M₄ receptor subunit has no obvious functional role in the mouse bladder as demonstrated by the inability of its antagonist, PD 102807 to modify the contractile effect of either CCh or EFS, an observation which is consistent with the findings of Choppin and Eglen, (2001b).

The response to ATP suggests heterogeneous distribution of P2X receptors in the mouse bladder. This fact was substantiated by the failure of α,β,methyleneATP-induced-P2X desensitization to completely abolish the contractile response to ATP. It has been shown that P2X receptor subtypes can be distinguished by α,β,methyleneATP (Dunn et al., 2001 review). P2X₁ and P2X₃ receptors have high affinity for α,β,methyleneATP and are desensitized rapidly, on the other hand P2X₂ and P2X₂/₃ receptor subtypes have low affinity for α,β,methyleneATP and are slowly desensitized (Dunn et al., 2001 review). Thus, it is conceivable that ATP responses that are resistant to α,β,methyleneATP-induced P2X desensitization were mediated by either the homomeric P2X₂ or the heteromeric P2X₂/₃ receptor subtypes or both. This finding is particularly interesting in that it suggests a post-junctional activity for these receptors, contradicting the current view that their distribution, except for P2X₁, is restricted to the intramural nerves (Cockayne et al., 2000; Vlaskovka et al., 2001; Rong et al., 2002; Cockayne et al., 2005). It is therefore possible that some of these receptors are expressed either on the urothelium or the detrusor muscle. While it is necessary to further investigate the functional implication of these observations it would be reasonably to speculate that if these receptors are located post-junctionally their up regulation could contribute to the pathogenesis of bladder dysfunction. In fact, in detrusor biopsy of patients with idiopathic detrusor instability, P2X₂
mRNA was found to be significantly elevated while other P2X receptor subtypes were significantly decreased. In addition, a purinergic component of nerve mediated contractions could be found in specimens of unstable human bladders, although not in the normal controls (O’Reilly et al., 2002).

In contrast to the response to ATP, bradykinin produced a classic monophasic dose response curve with a high potency (EC$_{50}$ 90nM). It also enhanced the response to neuronally mediated bladder contractions. These findings agree with previous evidence that bradykinin acts by sensitizing the primary afferent nerves either through release of sensory neuropeptides from nerve terminals (Marceau et al., 1980; Maggi et al., 1989) or by direct stimulation of bladder afferent nerves (Lecci et al., 1995). It is therefore reasonable to suggest that bradykinin potentiation of neuronally mediated bladder contraction seen in this model is due to the sensitization of intramural nerves, and could thus mimic the pathophysiological process involved in chronic inflammation. A possible mechanism is that the activation of nociceptive nerves by bradykinin evokes the release of tachykinins from axon collaterals and in turn enhances the sensitivity of the smooth muscle to stimuli (Marceau et al., 1980).

As well as evoking excitatory responses, exogenous application of bradykinin also evoked tachyphylaxis following an initial rise in intravesical pressure. In the excitatory phase bradykinin potentiated electrically evoked bladder contraction peaking after five minutes of continuous exposure. Previous studies have reported that the pharmacological effects of bradykinin can be attributed to two main receptors: B$_1$ and B$_2$ (Regoli and Barabe, 1980). The B$_1$ receptor is thought to be stimulated by des-Arg9-Leu8-bradykinin, while the B$_2$ receptor is stimulated by bradykinin. Bradykinin B$_2$ receptors are constitutively expressed by various cell types (Bhoola et al., 1992) and it seems to be the predominant receptor mediating contractile responses under
normal conditions (Meini et al., 2000). HOE 140 has been shown to selectively inhibit the action of bradykinin on B2 receptors in a non competitive manner (Wirth et al., 1991; Meini et al., 2000). On the other hand, the kinin B1 receptor is reported to be expressed de novo after inflammatory stimuli or tissue injury (Marceau et al., 1998; Belichard et al., 1999; Wotherspoon and Winter, 2000) and can be antagonized by desArg10 HOE 140, a selective B1 antagonist. Consistent with this we found that HOE 140 (1µM) almost completely suppressed bradykinin induced contraction, and considerably reduced bradykinin-induced potentiation of EFS mediated bladder contraction while desArg10 HOE 140 (1µM) had no significant antagonistic effect on either parameter. These findings would suggest that in the mouse whole bladder, the effects of bradykinin were mediated by the constitutively expressed B2 receptor, an observation which is in line with the proposal of Meini et al. (2000) in human and rat urinary bladder. The fact that HOE 140 also modulated direct contraction of the bladder means that B2 receptors are present on tissues other than the intramural nerves. It could be that they are also expressed in the muscle and urothelium as recently published by Chopra et al. (2005), who found B2 receptor mRNA to be constitutively expressed in the muscle and urothelium of normal rat bladders.

Since bradykinin had little effect on the bladder response to either ATP or carbachol, it is reasonable to postulate that bradykinin-induced potentiation of the response to EFS is through facilitation of parasympathetic neurotransmission. We therefore tested this hypothesis by examining the effect of bradykinin on the neurogenic response on the cholinergic and purinergic components for any differential effect. The sensitizing effect of bradykinin does not appear to be due to enhanced release of acetylcholine, since atropine, although reducing the size of the response to EFS, failed to modify either the direct bradykinin-induced bladder contraction or the potentiation of intramural nerve stimulation. Similar observations were reported by Downie et al.
(1981) and Nakahata et al. (1987) using rabbit detrusor strips. It would therefore seem that bradykinin sensitization of the neurogenic responses is mediated through purinergic transmission. Compatible with this notion, α,β,methyleneATP, although it substantially reduced the size of the response to EFS, also markedly enhanced the bradykinin-induced potentiation of EFS without modifying the direct bradykinin-induced bladder contraction. Acevedo et al. (1990) and Patra et al. (1996), similarly observed the inhibitory effect of α,β,methyleneATP on EFS in rat and guinea-pig detrusor strips respectively.

The bladder response to intramural nerve stimulation remaining after desensitization may indicate that the fraction of receptors resistant to α,β,methyleneATP desensitization on the intramural nerves are likely to be responsible for the enhancement effect of bradykinin-induced potentiation of EFS. In addition, these subset of receptors may be identified as either the homomeric P2X2 or the heteromeric P2X2/3 receptors or both subtypes since P2X2 purinoceptor subtypes are known to have low affinity for α,β,methyleneATP and slowly desensitize as indicated earlier (Dunn et al., 2001 review).

In conclusions our results show that the mouse bladder behaves in a similar manner to other mammalian bladders, and the effects of bradykinin suggest that it possesses both pre and post junctional activity. Its prejunctional action is facilitated by the release of ATP from nerve terminals, and its postjunctional action is mediated by the B2 receptor. The mouse isolated whole bladder set-up appears to be an effective functional assay to investigate the mechanisms underlying bladder pain and overactivity, and therefore may serve as a powerful tool in the aid of developing therapeutic agents for the treatment of bladder dysfunction.
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FOOT NOTE

We wish to thank Abbot Laboratories for financial support.
LEGENDS FOR FIGURES

Fig. 1 Experiment to establish isovolumetric-pressure relationship in isolated mouse whole bladder. Graph represents the pressure-volume response curve, to determine the range of volumes over which optimum responses to electrical field stimulation is produced. The curve was constructed by cumulatively filling the bladder by increments of 0.02ml until a final volume of 0.2ml was reached. The bladder was filled at the rate of 0.8ml/hr, intermittently interrupted (at every increment of 0.02ml) to electrically stimulate the bladder. The cycle recommence each time the bladder response had returned to the preceding baseline level. Contraction is reported as mean intravesical pressure ± SEM. EC₅₀ is used as a measure of potency.

Fig. 2 Experiments to establish optimum TTX -sensitive EFS parameters in isolated mouse whole bladder. Effect on intravesical pressure response of increasing pulse width PW (a), train duration TD (b), voltage V (c) and frequency Hz (d) at the constant parameters indicated in the body of individual graphs. Experiments were conducted by transmural electrical depolarization of bladder nerve fibres by varying one parameter while the others are kept constant. At the end of each stimulatory response the preparations were allowed to return to baseline before repeating the procedure. Frequency response curves in the absence and presence of TTX (0.3μM) were also constructed (d). Contraction was reported as mean intravesical pressure ± SEM.
Fig. 3 Experiments to establish whether isolated mouse bladder preparations responses to transmural nerve stimulation consists of two components, the cholinergic component mediated M3 receptors and the purinergic component mediated by P2X receptors as demonstrated in rat and guinea-pig (Brading and Williams, 1990). These experiments are in validation of the isolated mouse whole bladder. Frequency response curve in the isolated mouse whole bladder model (a) and isolated mouse muscle strips: in this graph n represent number of strips while N is the number of animals (b). The bladder was stimulated by transmural electrical depolarization of its nerve fibres using the parameters specified in the body of the graphs while varying the frequency (1-30Hz) at 5 min intervals. After obtaining control responses, the rest of the recordings were carried out in the presence of either atropine (1µM) or combined atropine and two treatments of α,β,methyleneATP (10µM) or in the presence of TTX (0.3µM). Contact period of 20 min was used. Contraction is reported as mean intravesical pressure ± SEM.

Fig. 4 Experiments to characterize muscarinic receptors in isolated mouse whole bladder. Summary graph of the effects of a range of muscarinic receptor antagonists, pirenzepine, AF-DX 116, 4-DAMP and PD 102807 selective for M1, M2, M3 and M4 respectively on either CCh or EFS-induced bladder contractions. The experiments were carried out by recording bladder responses to either CCh (3 µM) or EFS before and after 20 minutes exposure to either pirenzepine (0.3µM), AF-DX 116 (1µM), 4-DAMP (10nM), PD 102807 (1µM) or the vehicle (DMSO). Results are expressed as % change from the control response and significance was assessed using ANOVA followed by Fisher’s test. n=4/antagonist or vehicle. Significant difference between data was assumed with *p< 0.05, **p<0.01 or ***p<0.001. Contraction is reported as mean intravesical pressure ± SEM.
Fig. 5 Experiments to characterize purinoceptors in isolated mouse whole bladder.

In an attempt to confirm whether the distribution of P2X receptors are heterogeneous in nature as would be suggested by the biphasic response curve of ATP, the effect of $\alpha,\beta$-methyleneATP induced-desensitization of P2X receptors on intravesical pressure response to ATP was investigated. P2X-desensitization experiment was carried out by recording the response of ATP before and after treatment with $\alpha,\beta$-methyleneATP (a). After obtaining ATP control responses the tissue was washed out, and thereafter subjected to three consecutive treatments with $\alpha,\beta$-methyleneATP, the bladder responses after each treatment being allowed to return to pretreatment level before applying the next dose. Graph represents summary response (b). Results were assessed using ANOVA followed by Fisher’s test. Significant difference between data was assumed with *$p<0.05$, **$p<0.01$ or ***$p<0.001$. Contraction is reported as mean intravesical pressure $\pm$ SEM.

Fig. 6 Evaluation of bradykinin effect on intramural nerve stimulation in isolated mouse whole bladder. Graph of bradykinin dose response curve (a). Graph of frequency response curve of mouse whole bladder infused with bradykinin intravesically (b). FRC was constructed in the absence and presence of TTX in bladders filled intravesically with either saline or bradykinin (1$\mu$M). The bladder was stimulated by transmural electrical depolarization of its nerve fibres with 0.2ms duration, 50V, and varying frequency (1-30Hz, at multiples of 5Hz) for 3s train duration. Results were assessed using ANOVA followed by Fisher’s test. Significant difference between saline solution and the other groups was assumed with *$p<0.05$, **$p<0.01$ or ***$p<0.001$. Contraction is reported as mean intravesical pressure $\pm$ SEM.
Fig. 7 Time-course of the effect of external application of bradykinin on intramural nerve stimulation in isolated mouse whole bladder. The aim was to find the optimal exposure time at which bradykinin produced the most marked potentiation of the response to intramural nerve stimulation (EFS). Effect of two different concentrations of bradykinin (10nM & 100nM) on electrically evoked bladder contractions (a). Representative trace of the effect of electrically evoked bladder contractions pre and post five minutes bradykinin (10nM) contact period (b).

Fig. 8 Summary graph of bradykinin effects in the presence of either Krebs’ solution, HOE 140 (1µM), desArg^{10}HOE 140 (1µM), atropine (1µM) or α,β-methyleneATP (30µM) on bradykinin-induced potentiation of bladder response to EFS. Results were assessed using ANOVA followed by Fisher’s test. Significant difference between Krebs’ solution and the other groups was assumed with *p< 0.05, **p<0.01 or ***p<0.001. Contraction is reported as mean intravesical pressure ± SEM.

Fig. 9 Experiments to examine the influence of bradykinin on ATP or CCh-induced bladder contractile response Effects of bradykinin on either ATP, n = 5 (a) or CCh-induced bladder contraction, n = 4 (b). Intravesical pressure responses to ATP (3mM) or carbachol (3µM) were recorded before and after treatment with bradykinin (10nM). Drugs were washed out by continuous overflow as soon as the maximum response was attained to avoid desensitization, except in the case of bradykinin, which was not washed out until after the ATP or carbachol test response.
Fig. 10 Experiment to evaluate the effect of atropine or α,β,methyleneATP on bradykinin-induced bladder muscle contraction and on potentiation of EFS mediated bladder contraction. Traces demonstrate the effect of atropine (1 µM), n = 2 (a) or α,β,methyleneATP (30 µM), n = 3 (b) on bradykinin (10 nM) induced-bladder contraction and on bradykinin-potentiation of the response to EFS (0.2 ms pulse duration, 3 s or 35 s train duration, supramaximal voltage 50 V and 20 Hz frequency). For this set of experiments, on obtaining EFS control responses, further EFS recordings were made after exposure to either atropine or α,β,methyleneATP alone and after consecutive treatment with bradykinin. For atropine, tissues were exposed at least for 20 minutes before making the first EFS recording, while for α,β,methyleneATP the first EFS was made after the bladder response had returned to baseline (b).
FIG 1

% of Maximum Intravesical Pressure Response

Volume (ml)

0 0.02 0.06 0.1 0.14 0.18

0 20 40 60 80 100 120

n = 4

Intravesical Pressure Response
a)

**FIG 2**

- **% of Maximum Intravesical Pressure**
- **Pulse Width (PW)**
- **n=6**
- **constant: TD 3s, 20Hz & 50V**

Time (ms) vs. % of Maximum Intravesical Pressure graph with data points and a curve fitted to the data. The graph shows the relationship between pulse width and time, with specific conditions for the experiment.
b)

FIG 2

% of Maximum Intravesical Pressure

Time (s)

n=6
constant: PW 0.2ms, 20Hz & 50V
% of Maximum Intravesical Pressure vs. Voltage (V)

- VOLTAGE (V)

n=6
constant: PW 0.2ms, TD 3s, & 20Hz

FIG 2
FREQ PRE TTX
FREQ POST TTX (0.3µM)

n=6
constant: PW 0.2ms, TD 3s & 50V

% of Maximum Intravesical Pressure
Frequency (Hz)

FIG 2
FIG 3

% of Maximum Intravesical Pressure vs. Frequency (Hz)

- Control
- Atropine (1 µM)
- Atropine + α,β,methyleneATP (10 µM)
- TTX (0.3 µM)

n=5

constant: PW 0.2ms, TD 3s & 50V
FIG 3

% of Maximum Contractile Response

% of Maximum Contractile Response (tension)

Frequency (Hz)

- Control
- Atropine (1µM)
- Atropine + α,β,methyleneATP (10µM)
- TTX (0.3µM)

n=9/N=3
constant: PW 0.05ms, TD 2s & 50V
% Change from Control

Veh PRZPN AF-DX116 4-DAMP PD 102807

n = 4

EFS: PW 0.2ms, TD 3s, 20Hz & 50V

CCh (3μM)

***

**

*
Intravesical Pressure Response (cmH2O)

FIG 5

- ATP (3mM) Pre α,β,methyleneATP
- ATP (3mM) Post α,β,methyleneATP

Treatment Groups

n=3

**
FIG 6

% of Maximum Intravesical Pressure Relative to Carbachol

Concentration (M)

Bradykinin

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FIG 6

% of Maximum Intravesical Pressure Relative to Carbachol

- Bradykinin (1μM)
- Saline solution
- TTX (0.3μM)

n=6
constant: DURATION 3s, PW 0.2ms & 50V

FIG 6

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Published on August 30, 2006 as DOI: 10.1124/jpet.106.108902
**FIG 7**

* EFS: PW 0.2 ms, TD 3s, & 50V

Bradykinin (10nM)
% Potentiation of EFS (Intravesical Pressure Response)

Treatment Groups

Krebs' solution
HOE140 (1µM)
Atropine (1µM)
α,β-methyleneATP (30µM)

desArg10 HOE140 (1µM)

n=7

**

***
FIG 9
FIG 9

b)

- CCh (3μM)
- BK (10nM), 5 min Contact Period
- Washout Point
- CCh = Carbachol
- BK = Bradykinin
- Post BK
**FIG 10**

- **Atropine (1μM), 30 min Contact Period**

- **BK = Bradykinin**
- **EFS: PW 0.2ms, TD 35s, 20Hz & 50V**

- **BK (10nM), 5 min Contact Period**
**FIG 10**

BK = Bradykinin

* EFS: PW 0.2ms, TD 35s, 20Hz & 50V

α,β,methyleneATP (30μM)

BK (10nM), 5 min Contact Period