P2X RECEPTORS AND THEIR ROLE IN FEMALE IDIOPATHIC DETRUSOR INSTABILITY

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ABSTRACT

Purpose: It is clear from previous studies that adenosine triphosphate is released as a contractile co-transmitter with acetylcholine from parasympathetic nerves supplying the mammalian bladder but the physiological significance of ligand gated purinergic P2X receptors in human bladder innervation has not been adequately investigated. We examined the role of these receptors in female patients with idiopathic detrusor instability.

Materials and Methods: Female patients with idiopathic detrusor instability were recruited for cystoscopy and bladder biopsy with ethics approval. Control tissue was obtained from age and sex matched patients with a urodynamically proved stable bladder. We obtained 4 bladder biopsies per patient from the posterior wall. Samples were analyzed in an organ bath for functional studies of the detrusor muscle to assess the purinergic contribution to its contraction. In addition, we performed quantitative analysis using reverse transcriptase-polymerase chain reaction and immunohistochemical localization of P2X receptors.

Results: In patients with idiopathic detrusor instability detrusor $P2X_2$ receptors were significantly elevated, while other P2X receptor subtypes were significantly decreased. A purinergic component of nerve mediated contractions was not detected in control female bladder biopsy specimens but there was a significant component in unstable bladder specimens. It was particularly prominent at stimulation frequencies of 2 to 16 Hz. which are likely to be most relevant physiologically. Approximately 50% of nerve mediated contractions were purinergic in idiopathic detrusor instability cases.

Conclusions: In patients with idiopathic detrusor instability there is abnormal purinergic transmission in the bladder, which may explain symptoms. This pathway may be a novel target for the pharmacological treatment of overactive bladder.

KEY WORDS: bladder; adenosine triphosphate; receptors, purinergic; biopsy; electric stimulation

Urinary incontinence is increasingly acknowledged as a major health issue. However, many patients and unfortunately many health professionals assume that this incontinence is a natural consequence of aging and childbirth. The scale of this problem is shown by the estimated 200 million individuals worldwide who have problems associated with urinary incontinence.[1] A common cause of urinary incontinence is detrusor instability or overactive bladder, which affects some 50 to 100 million people.[2] It has a major impact on quality of life and leads to considerable economic costs. For instance, the latest pharmacotherapy for detrusor instability, namely tolterodine, currently grosses more than $600 million yearly. A recent review showed that 17% of European adults have 1 or more symptoms of overactive bladder.[2] Despite its prevalence the etiology of idiopathic detrusor instability is poorly understood.

Recent evidence indicates that changes in bladder innervation as a result of increased neurotrophin production by detrusor smooth muscle are associated with bladder overactivity and a highly effective stimulator of nerve growth factor production in human bladder smooth muscle cells is adenosine triphosphate, implying a role for adenosine triphosphate as a potential initiator of unstable bladder.[3] Atropine resis-

tance of a proportion of parasympathetic bladder innervation was recognized as long ago as 1895 by Langley and Anderson.[4] Many subsequent studies have supported this finding, leading to the conclusion in 1970 of a nonadrenergic noncholinergic transmitter innervating the bladder.[5] In 1972 Burnstock et al postulated that this nonadrenergic noncholinergic transmission was in fact purinergic, involving adenosine triphosphate release.[6] This conclusion is now widely accepted to be the case in a wide variety of animals.[7] In the human bladder there is also now much evidence for the existence of purinergic signalling, especially in some pathological conditions.[8]

Therefore, in this study we examined the hypothesis that abnormal purinergic transmission contributes to the etiology of idiopathic detrusor instability. Since to our knowledge there are no animal models of this condition, we have obtained and analyzed bladder biopsy specimens from patients with idiopathic detrusor instability and matched controls. We performed polymerase chain reaction (PCR) to determine quantitative measures of the expression of all 7 known members of the family of adenosine triphosphate ligand gated ion channels, the ligand gated purinergic or P2X family. We also studied the immunohistochemical distribution of P2X receptor subtypes and the functional responsiveness of idiopathic detrusor instability versus control bladders to chemical and electrical stimulation.

MATERIALS AND METHODS

We recruited into our study 20 female patients 35 to 75 years old (mean age 51.8) diagnosed with idiopathic detrusor instability after standard dual channel subtracted cystometry. In these women idiopathic detrusor instability was diagnosed based on history, normal neurological examination and urodynamics. At least 1 type of anticholinergic medication and bladder retraining had been attempted with little improvement. Cystoscopy was performed to rule out a possible bladder pathology cause of symptoms, such as calculus. Findings were normal in each recruited patient. At the same time 20 age and sex matched controls 35 to 71 years old (mean age 53.6) were recruited, in whom urodynamics showed a stable bladder. These patients underwent screening urodynamics before surgical treatment of genuine stress incontinence via colposuspension. To assess the contractile response of human detrusor to potassium chloride we also obtained bladder biopsies from 7 male controls undergoing a routine urological endoscopic procedure.

All patients with idiopathic detrusor instability were admitted to the hospital for inpatient cystoscopy and biopsy using general anesthesia with approval of the local ethics committee. Tissue from female controls was obtained in the same manner as that in patients with idiopathic detrusor instability immediately after colposuspension. Four bladder biopsies approximately 3 mm. in diameter, including urothelium and the underlying detrusor, were consistently obtained from the posterior wall of the bladder just above the trigone with Storz cold cup biopsy forceps.

Immunohistochemical testing. A single biopsy per patient was embedded in OCT compound, oriented to enable transverse sectioning and frozen in isopentane pre-cooled in liquid nitrogen. Tissues were then sectioned at 12 μm. using a cryostat, thaw mounted onto microscope slides and air-dried at room temperature. Slides were stored at $-20C$ and allowed to return to room temperature for at least 10 minutes before use.

Primary Antibodies: Polyclonal antiserum was raised in rabbits using keyhole limpet hemocyanin conjugated immunogens, which were peptides corresponding to $P2X_{1\text{ to }7}$ subunit specific 15 amino acid C-terminal sequences.[9] Antibody specificity was verified by immunoblotting with membrane preparations from CHO-K1 cells expressing the cloned $P2X_{1\text{ to }7}$ receptors.[9] Antibodies recognized only a single protein band of the expected size and were shown to be receptor type specific.

Diaminobenzidine Enhancement Immunostaining: The avidin-biotin-peroxidase complex technique used was described by Llewellyn-Smith et al.[10, 11] Slide mounted sections were thawed and fixed in 4% formaldehyde and 0.2% picric acid solution in phosphate buffered saline (PBS) for 2 minutes. To activate endogenous peroxidase sections were treated with 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Nonspecific protein binding sites were blocked by a 20-minute incubation with 10% normal horse serum in PBS containing 0.05% methiolate. P2X antibodies were diluted to 2.5 and 5 μg./ml., determined as optimal by previous experimentation, with 10% normal horse serum and incubated for 24 hours at room temperature. The secondary antibody was a biotinylated donkey anti-rabbit IgG diluted 1:500, in 1% normal horse serum-PBS-methiolate for 30 minutes, followed by peroxidase conjugate, diluted 1:1,000, in PBS-methiolate for 30 minutes.

To visualize the reaction product the nickel-diaminobenzidine enhancement technique was used. Controls were performed by omitting the primary antibody. Immunostaining was observed using high definition light microscopy.

Fluorescence Immunostaining: This technique was used to double stain the sensory specific purinoceptor $P2X_3$ and pan-neuronal marker protein gene product 9.5 to assess colocalization. Slides were thawed and specimens were post-fixed, as described. Nonspecific protein binding sites were blocked by 10% normal goat serum for 40 minutes. Rabbit polyclonal $P2X_3$ receptor antibody, diluted 1:500 and 1:1,000 in PBS containing 0.2% Triton X-100, was applied to the specimens for 48 hours of exposure. Sections were then incubated with certain substances diluted in PBS and 0.2% Triton X-100, including biotin-conjugated anti-rabbit antibody raised in goat, 1:400, for 90 minutes, avidin-biotin-peroxidase complex reagents (the avidin-biotin reaction) for 30 minutes, biotinyl tyramide, 1:75, for 8 minutes, fluorescein isothiocyanate conjugated extra-avidin, 1:500, for 2 hours and rabbit polyclonal protein gene product antibody, 1:1,000, for 24 hours; followed by TRITC conjugated rabbit antibody raised in goat, 1:200, for 2 hours. Slides were then mounted and covered with coverglasses. Immunostaining was visualized by fluorescence microscopy.

Quantitative reverse transcriptase (RT)-PCR. A single biopsy sample per patient was snap frozen in liquid nitrogen and stored at $-70C$ until experiments were done. To compare various genes amplification efficiency of the primer-probe sets was adjusted using standard curves (see table). Equivalent mass amounts were determined in each standard tube at an optical density of 260 nm. Without such standard curves cross comparison cannot be performed since the cycle threshor values obtained may also reflect different amplification efficiencies. This technique has been described previously.[12]

Data generated at each run were analyzed using computer software, normalized with the 18S ribosomal RNA endoge-

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Oligonucleotide sequence details of all P2X and normalization control primers and probes

P2X1:	
Forward	5′-GCGTAATAAGAAGGTGGGCGTTA-3′
Reverse	5′-GCCGCTCGAGGTCTGGTA-3′
Probe	5′-FAM-AACACCCACCCGATGACGTAGACCA-TAMRA-3′
P2X2:	
Forward	5′-CAGGTTTGCCAAATACTACAAGATCA-3′
Reverse	5′-AACTTCCCGGCCTGTCCAT-3′
Probe	5′-FAM-AATGCGCGGATCCCGTAGGCCTTGA-TAMRA-3′
P2X3:	
Forward	5′-TCTTCACCTATGAGACCACCAAGTC-3′
Reverse	5′-GATCAGAAGCTGAACTACTCGGTTGATG-3′
Probe	5′-FAM-TGGTTGTGAAGAGCTGGACCATC-TAMRA-3′
P2X4:	
Forward	5′-CGCAGGACACGGTTTCCA-3′
Reverse	5′-GCAGTCCCAGTTGACCTGGAT-3′
Probe	5′-FAM-ATGGCCGTGGAGGGAGGCATC-TAMRA-3′
P2X5:	
Forward	5′-CGCTGGGGAAGCGGTTA-3′
Reverse	5′-GCACCAGGCAAAGATCTCACA-3′
Probe	5′-FAM-ACGGAGTGAAGACCGGCCGCT-TAMRA-3′
P2X6:	
Forward	5′-CACTGCCGCTATGAACCACAA-3′
Reverse	5′-CGAAGGTCCCTCCAGCCTT-3′
Probe	5′-FAM-CCCCTACTGTCCCGTGTTCCGCA-TAMRA-3′
P2X7:	
Forward	5′-CTTTCTCAAAACAGAAGGCCAAGA-3′
Reverse	5′-CAACCTCGGTCAGAGGAACAGA-3′
Probe	5′-FAM-TGTGTCCCGAGTATCCCACCCGC-TAMRA-3′
18S rRNA:	
Forward	5′-CGGCTACCACATCCAAGGAA-3′
Reverse	5′-GCTGGAATTACCGCGGCT-3′
Probe	5′-JOE-TGCTGGCACCAGACTTGCCCTC-TAMRA-3′
Calponin:	
Forward	5′-TGTTTGAGAACACCAACCATACACA-3′
Reverse	5′-GTTTCCTTTCGTCTTCGCCAT-3′
Probe	5′-FAM-TCCACCCTCCTGGCTTTGGCC-TAMRA-3′
Smoothelin:	
Forward	5′-GCTGATGAAGGCGCAGAGTCT-3′
Reverse	5′-CTCCTTCTCCAGCTTCTCAATCAT-3′
Probe	5′-FAM-AAGACCTCAGCCTCCCAGGCGC-TAMRA-3′
β-actin:	
Forward	5′-TCACCCACACTGTGCCCATCTACGA-3′
Reverse	5′-CAGCGGAACCGCTCATTGCCAATGG-3′
Probe	5′-FAM-ATGCCCT-TAMRA-CCCCCATGCCATCCTGCGT-3′

nous control and calculated as copies per 10 ng. total RNA. Standard curves were used for 18S ribosomal RNA and P2X genes. We prepared 10-fold dilutions of 10 pg. to 0.1 fg. from total RNA or plasmids containing the various P2X subtypes. Since standard curves were generated per subtype, variation in PCR efficiency should not have been a factor when cross comparing results among subtypes. After RT-PCR the data were analyzed with the threshold set at 0.08. Since values were normalized to a 10 ng. total RNA equivalent, all results were calculated as copies per 10 ng. total RNA. To compensate for sample-to-sample variation in smooth muscle content these values were further normalized to calponin and smoothelin, which are smooth muscle specific markers. Statistical comparison was done using a statistical software package.

Functional experiments. Two biopsy samples per patient were immediately transported in modified Krebs solution at ambient temperature. Biopsy samples were dissected under microscopy to obtain muscle strips approximately 3 to 4 mm. long. Silk ligatures were applied to each end of the strip. An end was applied to a rigid support and the other to a force displacement transducer. The strip was then placed in a 10 ml. organ bath containing Krebs solution continuously gassed with 95% O_2-5% CO_2. This modified Krebs solution was composed of 133 mM. NaCl, 4.7 mM. potassium chloride, 16.4 mM. sodium bicarbonate, 0.6 mM. magnesium sulfate, 1.4 mM. NaH_2PO_4, 7.7 mM. glucose and 2.5 mM. $CaCl_2$, pH 7.2, at a mean plus or minus standard deviation of $37 \pm 0.5C$. Mechanical activity was recorded on a recorder. An initial tone of 0.5 gm. was applied and samples were allowed to equilibrate for not less than 90 minutes before the start of the experiment. Electrical field stimulation of the detrusor samples was applied by 2 platinum wire rings on the rigid support 2.5 mm. in diameter and 1 cm. apart, through which the sample was mounted.

Frequency response curves were constructed for control and idiopathic detrusor instability samples at 100 V., 0.5 millisecond, 0.5 to 32 Hz. and 30 seconds of stimulation every 5 minutes. It was repeated 20 minutes after the application of pyridoxalphosphate-6-azophenyl-1′,4′-disulphonic acid (PPADS) or after desensitisation with 3 μM. α,β-methylene adenosine triphosphate repeated 3 times to block any purinergic neurotransmission. The frequency response curve was then repeated 20 minutes after the further addition of 3 μM. atropine to inhibit muscarinic receptor mediated neurotransmission. When any response to electrical field stimulation remained in the presence of atropine, the curve was repeated in the presence of 1 μM. tetrodotoxin for 20 minutes to identify
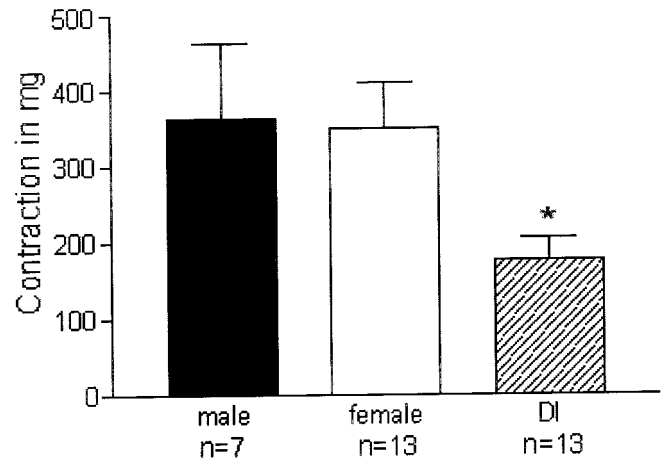

FIG. 1. Contraction of the detrusor muscle strips in response to standard concentration of 120 μM. potassium chloride. In *DI*, detrusor instability groups. Asterisk indicates significant at 5% level.

whether any of the response was due to direct stimulation of detrusor smooth muscle. For the second of each pair of samples concentration-response curves were constructed for 0.1 nM. to 300 μM. carbachol and P2X receptor agonists, including 1 μM. to 1 mM. adenosine triphosphate and 0.3 to 300 μM. β,γ-methylene adenosine triphosphate using single doses. Consecutive doses of carbachol were applied every 5 to 10 minutes, whereas consecutive doses of adenosine triphosphate and β,γ-methylene adenosine triphosphate were applied 15 to 20 minutes apart to protect against desensitisation. The contractile response to 120 mM. potassium chloride was then noted.

All frequency-response and concentration-response curves are expressed as the mean percent contraction of the 120 mM. potassium chloride biopsy sample contraction plus or minus standard error of mean to correct for differences in size and responsiveness of the biopsy samples. Statistical significance was tested by 2-way analysis of variance (ANOVA) using computer software to determine whether the frequency and concentration-response curves were significantly different. The value of $-\log EC_{50}$ were calculated from the carbachol concentration-response curves. Because the concentration-response curves to β,γ-methylene adenosine triphosphate and adenosine triphosphate did not achieve a maximum, we did not calculate these values. Statistical significance of the $-\log EC_{50}$ values for carbachol was tested by
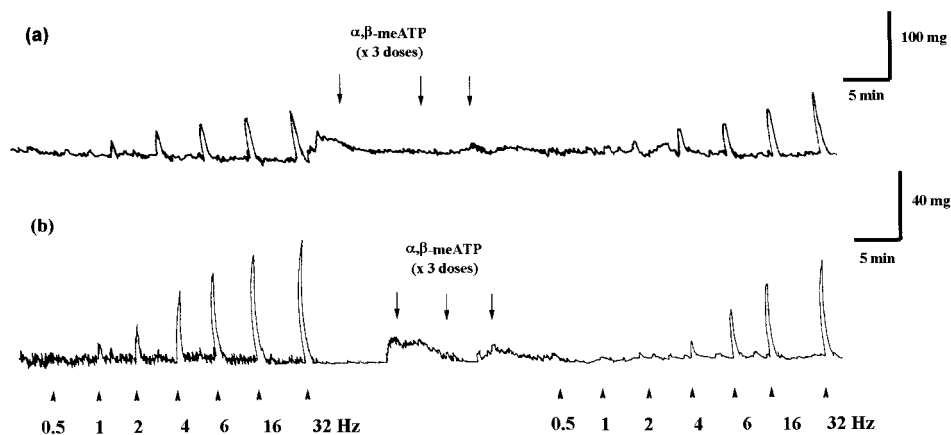

FIG. 2. Typical tracings of neurogenic contractions on human bladder biopsy samples. Typical frequency-response tracings. *a*, in female control bladder sample using same parameters as in fig. 1 there was no change in contractility after desensitization and all responses were tetrodotoxin sensitive. *b*, in idiopathic detrusor instability bladder sample at 100 V., 0.3 milliseconds, 0.5 to 32 Hz. and 30 second stimulation every 5 minutes before and after desensitization of P2X receptors with 3 doses of 3 μM. α,β-methylene adenosine triphosphate (α,β-meATP) note significantly decreased contractility after desensitization, showing significant purinergic component to innervation.
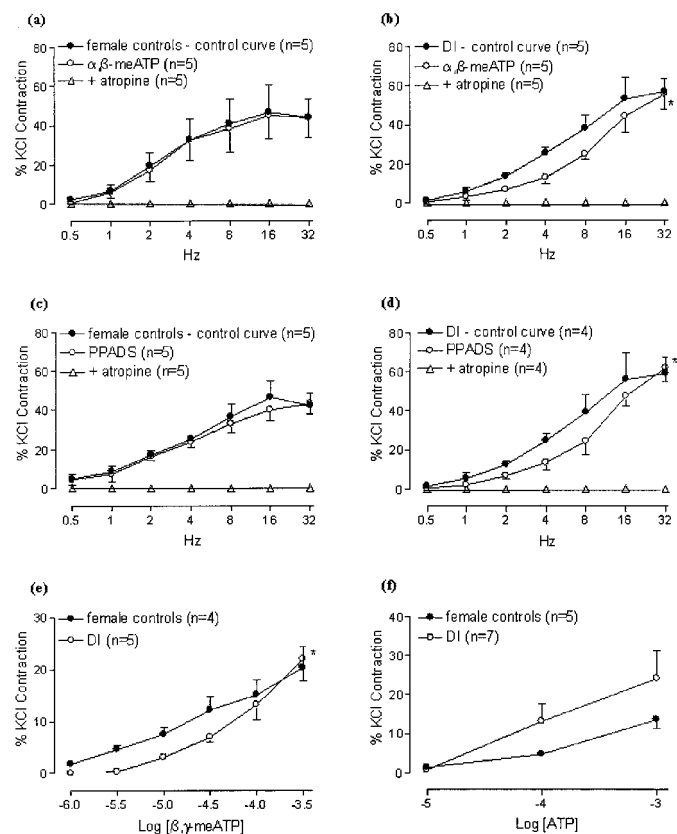
FIG. 3. *a*, electrical field stimulation of control detrusor at increasing frequencies of 0.5 to 32 Hz. before (filled circles) and after desensitization with α,β-methylene adenosine triphosphate (α,β-*meATP*), and after subsequent addition of atropine. *KCl*, potassium chloride. *b*, electrical field stimulation of idiopathic detrusor instability (*DI*) detrusor at 0.5 to 32 Hz. before and after desensitization with α,β-methylene adenosine triphosphate (α,β-*meATP*), and after subsequent addition of atropine. Note significantly decreased response after adding α,β-methylene adenosine triphosphate. Asterisk indicates 2-way ANOVA $p < 0.05$. *c*, electrical field stimulation of control detrusor before and after blocking with PPADS, and after subsequent addition of atropine. *d*, electrical field stimulation of the idiopathic detrusor instability detrusor before and after blocking with PPADS, and after subsequent addition of atropine. Note significantly decreased response after adding PPADS. Asterisk indicates 2-way ANOVA $p < 0.01$. *e*, concentration response curves of control and idiopathic detrusor instability detrusor to β,γ-methylene adenosine triphosphate shows significantly decreased response of idiopathic detrusor instability bladder. Asterisk indicates $p < 0.05$. *f*, concentration response curves of control and idiopathic detrusor instability detrusor to adenosine triphosphate shows increased response of idiopathic detrusor instability bladder, which was not significant on 2 way ANOVA.

the unpaired t test. For ANOVA and the paired t test significance was considered at $p < 0.05$.

RESULTS

Functional data. The peak detrusor contraction in response to a standard concentration of 120 mM. potassium chloride was measured in all samples and plotted by patient group (fig. 1). Samples from idiopathic detrusor instability cases showed significantly decreased contractions compared with male and female controls (unpaired t test $p < 0.05$), which in turn were not significantly different from each other. Idiopathic detrusor instability samples produced only about 60% of the force in controls. To correct for the difference in the contractility of detrusor samples in idiopathic detrusor instability cases and controls the response to electrical field stimulation and exogenously applied agonists are expressed as a percent of the potassium chloride contraction per tissue.

Using electrical field stimulation to activate the neuronal innervation of the detrusor we observed that control and idiopathic detrusor instability detrusor produced frequency dependent contractions (figs. 2 and 3). They were completely abolished by tetrodotoxin treatment. We examined the purinergic component in these responses by repeating electrical field stimulation after desensitisation with 3 doses of 5 μM. α,β-methylene adenosine triphosphate. In the control group desensitisation failed to inhibit the response to electrical field stimulation (figs. 2, *a* and 3, *a*), while the subsequent addition of 3 μM. atropine caused a complete blockage of electrical field stimulation responses. In contrast, α,β-methylene adenosine triphosphate desensitisation in idiopathic detrusor instability detrusor resulted in a significantly decreased response to electrical field stimulation (2-way ANOVA $p < 0.05$, figs. 2, *b* and 3, *b*). The subsequent addition of atropine abolished the remaining responses to electrical field stimulation. At intermediate electrical stimulation frequencies, which were likely to be the most relevant physiologically, approximately 50% of nerve induced contractions appeared to be purinergic.

We addressed the same question using 30 μM. of the nonspecific P2 receptor antagonist PPADS in various biopsy specimens. In controls this agonist failed to inhibit the response to electrical field stimulation (fig. 3, *c*), which again was completely atropine sensitive. In contrast, treatment of the idiopathic detrusor instability detrusor with 30 μM. of antagonist resulted in a significant decrease in the electrical field stimulation induced response (2-way ANOVA $p < 0.01$, fig. 3, *d*). Again the remaining responses were abolished by 3 μM. atropine. About 50% of the electrical field stimulation mediated contractions appeared to be purinergic at intermediate frequencies of stimulation by this methodology.

Furthermore, we examined the sensitivity of biopsy specimens to bath applied purinergic agonists. Adenosine triphosphate caused concentration dependent, transient contractions in the samples of controls and idiopathic detrusor instability cases (fig. 3, *f*). However, contraction magnitude was greater in idiopathic detrusor instability biopsies, consistent with the enhanced purinergic contractions with electrical field stimulation, although not significantly so. However, somewhat unexpectedly the adenosine triphosphate analog β,γ-methylene adenosine triphosphate elicited concentration dependent transient contractions but a lesser response in idiopathic detrusor instability than in control samples (2-way ANOVA $p < 0.05$, fig. 3, *e*). Because the response to β,γ-methylene adenosine triphosphate or adenosine triphosphate did not produce a maximum response in idiopathic detrusor instability or control samples, -log EC_{50} values were not calculated.

Purinoreceptor expression. Quantitative PCR: The copy number of P2X genes per 10 ng. total RNA was obtained per bladder tissue sample. We measured the detectable expression of $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_7$. $P2X_3$ and $P2X_6$ were expressed below the lower limit of detection of this assay. We also measured the absolute levels of expression of 2 markers of smooth muscle, calponin and smoothelin.

In the 20 control bladder tissue samples $P2X_1$ was the predominant purinoceptor subtype expressed, amounting to 57.5% of all P2X messages. The rank order of expression was $P2X_1 \gg P2X_4 > P2X_2 > P2X_7 > P2X_5 \gg P2X_3 = P2X_6 = 0$. Figure 4 shows the absolute expression levels. In the 20 patients with idiopathic detrusor instability P2X receptor expression differed markedly. $P2X_{1,4,5}$ and $_7$ were decreased by about 50% to 60% and these decreases were significant for $P2X_{1,4}$ and $_7$ (Mann-Whitney U test $p = 0.019$, 0.03 and 0.004, respectively, fig. 4, *a*). In contrast, $P2X_2$ was significantly increased in idiopathic detrusor instability bladders by about 50% (Mann-Whitney U test $p = 0.048$).

A potential complicating factor in the analysis of PCR data was that tissue composition may have varied in idiopathic
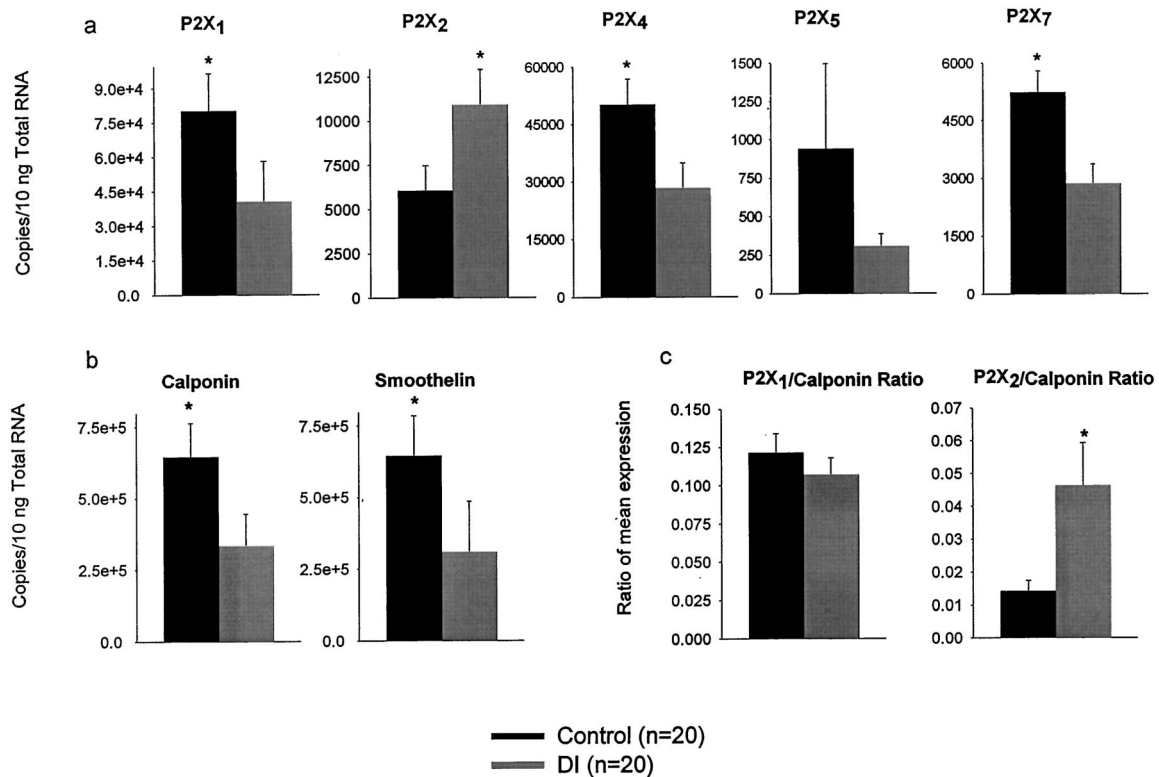
FIG. 4. Comparison of mean $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_7$ expression in control and idiopathic detrusor instability (*DI*) bladders on quantitative RT-PCR reveals significantly decreased $P2X_1$, $P2X_4$ and $P2X_7$ expression but significantly increased $P2X_2$ expression in idiopathic detrusor instability bladders. Asterisk indicates $p < 0.01$. *b*, comparison of mean calponin and smoothelin expression in control and idiopathic detrusor instability bladders demonstrates that they are significantly decreased by similar amount in idiopathic detrusor instability bladders. Asterisk indicates $p < 0.05$. *c*, comparison of mean $P2X_1$ and $P2X_2$-to-calponin expression in control and idiopathic detrusor instability bladders reveals that after normalization to account for intersample variation in smooth muscle content significantly increased $P2X_2$ expression was maintained. Asterisk indicates $p = 0.03$.

detrusor instability cases and controls. In fact, the lesser magnitude of potassium chloride induced contractions in idiopathic detrusor instability samples implies a lower smooth muscle component in this disease state. Therefore, we quantitatively measured calponin in all samples (figure 4, *b*) and noted an approximate 40% decrease in idiopathic detrusor instability samples (Mann-Whitney U test $p = 0.035$, fig. 4, *b*). This finding indicated decreased smooth muscle content in the unstable bladders. We also measured the smooth muscle marker smoothelin and its values closely reflected those of calponin with a significant decrease of about 40% in the from the idiopathic detrusor instability bladder samples versus controls (Mann-Whitney U test $p = 0.026$, fig. 3, *b*). $P2X_1$ is known to be localized to smooth muscle in the bladder, and so we also normalized its expression to calponin expression to account for the differences in smooth muscle content among samples. The ratio of $P2X_1$-to-calponin was similar in idiopathic detrusor instability and control samples, and did not differ significantly (Mann-Whitney U test, fig. 4, *c*). $P2X_2$ also appeared to be localized to vesical smooth muscle, and so we also expressed it as a function of calponin expression. As expected, in this context the $P2X_2$-to-calponin ratio was upregulated about 4-fold compared with controls (Mann-Whitney U test $p = 0.029$, fig. 4, *c*). Since other P2X subtypes are not expressed exclusively by smooth muscle cells in the bladder, they were not normalized to calponin.

Immunohistochemical Analysis: To localize P2X receptors in the human bladder we performed immunohistochemical study using antibodies against the 7 P2X receptors subunits.[9] Overall no major differences were apparent in the distribution of P2X immunoreactivity in bladder sections from detrusor instability cases or controls: $P2X_1$ immunoreactivity was clearly smooth muscle specific in the bladder biopsies of each tissue set and appeared localized to the cell perimeter (fig. 5, *a*). $P2X_2$ immunoreactivity was also largely restricted to vesical smooth muscle. However, unlike $P2X_1$, much staining appeared cytoplasmic rather than membrane bound (fig. 5, *b*). $P2X_3$ immunoreactivity was evident in nerve bundles running beneath the urothelium and in muscle layers (fig. 5, *c*). It was confirmed by double staining sections for $P2X_3$ and the pan-neuronal marker protein gene product 9.5 (fig. 6). $P2X_4$ immunoreactivity had distribution similar to that of $P2X_1$, mostly on smooth muscle and mostly apparently membrane bound (fig. 5, *d*). $P2X_5$ immunoreactivity was specifically located at the urothelium (fig. 5, *e*). $P2X_6$ and $P2X_7$ immunolabeling was not observed in the bladder.

DISCUSSION

The principal findings of our experiments are that purinoceptor expression and function are altered in patients with idiopathic detrusor instability. Therefore, abnormal purinergic transmission may explain the altered bladder motility in patients with idiopathic detrusor instability. Thus, drugs targeting P2X receptors may represent a novel and effective way of treating this disease.

The molecular cloning of 7 distinct P2X receptor subunits greatly increased our understanding of purinergic transmission generally and its potential role in bladder function particularly. Polyclonal antibodies to each receptor subtype have been generated,[7,9] which facilitated studies of receptor distribution and complimented earlier studies using Northern blot analysis and in situ hybridization of messenger (m)RNA.[13] However, previous immunohistochemical studies of P2X receptor distribution and autoradiographic studies of localization in the bladder have been limited and mostly
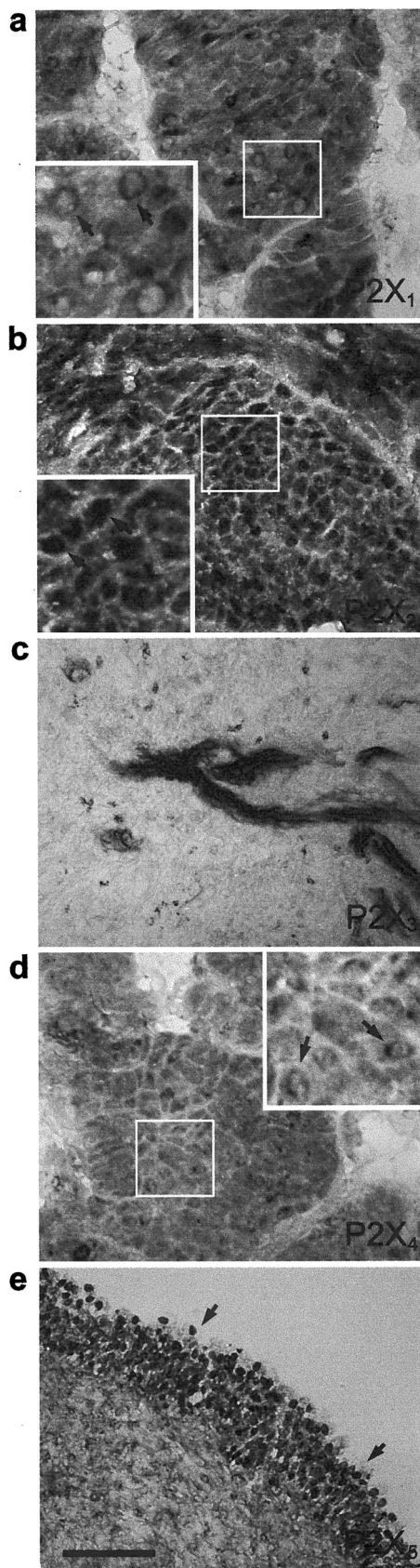
FIG. 5. Photomicrographs of immunohistochemical analysis of P2X receptors in 12 μm. transverse section bladder biopsies. *a*, smooth muscle membrane immunoreactivity of $P2X_1$ (arrows). *b*, $P2X_2$ immunoreactivity appears to be largely within intracellular compartment of smooth muscle (arrows). *c*, $P2X_3$ immunoreactivity on nerve bundle below urothelium. *d*, $P2X_4$ immunoreactivity on smooth muscle cell membrane (arrows). *e*, $P2X_5$ immunoreactivity in urothelial cells. Scale bar indicates 50 μm. (*a* to *d*) and 100 μm. (*e*).

confined to $P2X_1$ to 3.[14,15] Recently Lee et al described the distribution of all 7 P2X receptors in the rat bladder[16] and our findings in the human bladder are similar.

The immunohistochemical and PCR data that we present are broadly consistent with earlier findings, extending them in several important ways. The technique of real-time quantitative PCR has been proved to be rapid and sensitive for quantifying gene expression.[17] All experimental approaches imply that the predominant P2X receptor subtype in normal bladder is $P2X_1$, which is expressed mostly in detrusor smooth muscle.[16,18] Localization data do not enable assessment of whether the $P2X_1$ subunits form exclusively homomultimers.[19] It is indicated by immunolocalization studies as well as earlier Northern blot analysis, which have also shown the robust presence of $P2X_1$ mRNA in the bladder, as well as the PCR that we report.

The next most prevalent subtypes observed were $P2X_2$ and $_4$, which appeared to be largely localized to smooth muscle. Although their mRNA was expressed at significantly lower levels than $P2X_1$ transcripts, this finding raises the possibility of heteromeric assemblies of $P2X_{1/2}$ or $P2X_{1/4}$. These 3 subtypes accounted for more than 96% of all transcripts. The low level of $P2X_5$ and $_7$ did not appear to localize to smooth muscle, which confirms previous published studies in rat bladder.[16] Therefore, they are unlikely to make any significant contribution to motility regulation in normal bladders. The role of adenosine triphosphate for bladder sensory innervation is also important since transmitter release from peripheral sensory terminals in the bladder may also have a role in the efferent pathway by direct postjunctional effects on the detrusor.[20] In the 2 sets of bladder samples $P2X_3$ mRNA was below the detection, although the protein is expressed in the bladder, as demonstrated by immunohistochemical analysis. Its presence is evident in small caliber axons running predominantly in the suburothelium. Previously others have reported that they are sensory neurones.[21] Recent data on $P2X_3$ knockout mice indicate that adenosine triphosphate released at bladder distention activates these afferents, which in turn activates the afferent arm of the micturition reflex.[22]

We observed several marked quantitative differences in receptor expression in the biopsies of patients with idiopathic detrusor instability. However, a potentially complicating factor was that tissue composition may change in terms of smooth muscle content.[23,24] For this reason we also measured the expression of the 2 smooth muscle markers calponin and smoothelin. Calponin is known to be critically important for regulating smooth muscle contraction and it is considered a phenotypic smooth muscle cell marker.[25] The calponin content in phasic and tonic smooth muscle has been shown to be equivalent,[26] and so it would appear to be ideal as a marker of smooth muscle to enable comparisons of bladder samples. Calponin expression correlated highly with the other marker, smoothelin, implying that at least each is a marker of the same underlying structural features.[27] $P2X_{1, 4, 5}$ and $_7$ were decreased by 40% to 50% in idiopathic detrusor instability bladder samples. The decreases in $P2X_1$ and $_4$ were readily accounted for by the decreased smooth muscle content of these samples. However, $P2X_2$ expression was increased significantly in idiopathic detrusor instability samples, although we and Lee et al[16] noted that it was localized in smooth muscle. After normalizing for decreased smooth muscle content $P2X_2$ expression was greatly increased in idiopathic detrusor instability tissue. Thus, the increased functional responsiveness of idiopathic detrusor instability tissue to adenosine triphosphate was most readily explained by up-regulation of this receptor, although again it was not clear whether these subunits form homomeric or heteromeric functional receptors.

Our functional studies also showed the emergence of novel purinergic transmission in idiopathic detrusor instability
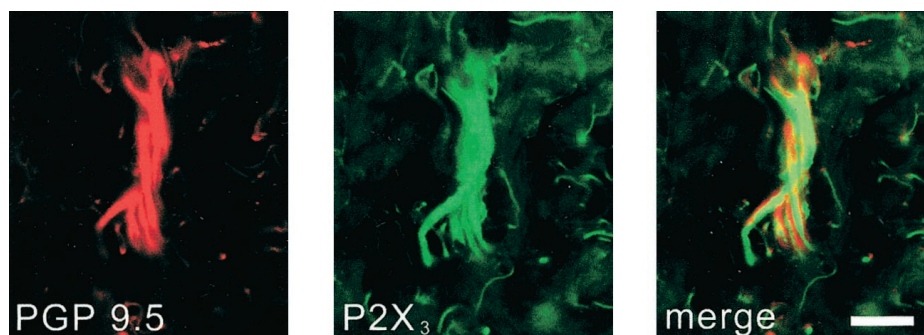
FIG. 6. Photomicrographs of 12 μm. transverse section bladder biopsies immunostained for pan-neuronal marker protein gene product (PGP) 9.5 (red areas) and P2X$_3$ (green areas), and merged to show P2X$_3$ and protein gene product 9.5 co-localization (yellow areas) in nerve fibers below urothelium. Scale bar indicates 25 μm.

bladder. We observed no detectable atropine resistant contraction in detrusor muscle from normal human bladders, although P2X receptors were clearly present. This finding has been shown in previous studies[28–31] and again recently by Bayliss et al.[8] However, there was significant atropine resistant contraction in the detrusor muscle of unstable bladders, which we showed is purinergic in origin.

Similar observations have been reported previously on purinergic signaling in other pathological bladder conditions[8,32,33] but study in idiopathic detrusor instability bladders is limited.[34]

Moreover, when comparing pathological with control human tissue, the criteria for entry to either group must be strictly defined. In our study of idiopathic detrusor instability in female patients each patient was recruited after standard urodynamic procedures performed by the same individual. Likewise control tissue was obtained from age and sex matched controls with a urodynamically proved stable bladder. Again the test was performed by the same individual. Previously others have evaluated tissue removed from cadaveric bladders as controls. Cadavers may be a good source of bladder tissue but they may not be completely appropriate because complete demographic and clinical details are not available and RNA degradation is a major difficulty in mRNA quantification. Our strategy of normalizing responses to the level of contraction induced by a standard dose of potassium chloride may also have enabled more meaningful comparisons since we identified different smooth muscle content on PCR as well as decreased potassium chloride induced contractile responses in idiopathic detrusor instability bladder tissue compared with female and male control bladders. Although idiopathic detrusor instability samples showed greater purinergic responses to electrical field stimulation, responses to the nucleotide agonist β,γ-methylene adenosine triphosphate were significantly attenuated. The reason for this apparent loss of sensitivity is not clear but may be associated with the loss of smooth muscle content in the tissue with its associated decreased P2X$_1$ subtype expression. It cannot be excluded that changes in P2X subunit expression in idiopathic detrusor instability bladders may allow the formation of different heteromultimeric forms with altered pharmacological properties, such as P2X$_1$ acting as part of a heteromultimer with P2X$_2$ or $_4$.

The presence of a significant purinergic component to electrical field stimulation in idiopathic detrusor instability bladders was demonstrated by the appearance of the desensitizing effects of α,β-methylene adenosine triphosphate and antagonistic effects of PPADS. The adenosine triphosphate analog α,β-methylene adenosine triphosphate has been used successfully in previous bladder studies to demonstrate purinergic input. It has been shown that this agonist binds, agonizes and desensitizes P2X$_1$ and $_3$ receptors.[35,36] In contrast, PPADS selectively and competitively antagonises P2X

receptor mediated contractions in guinea pig ileum, rabbit vas deferens[37,38] and rabbit bladder.[39] In our experiments PPADS caused a significant antagonistic effect on the contraction of idiopathic detrusor instability bladders compared with controls, again demonstrating the increased purinergic contribution in electrical field stimulation. In each of these experiments we used tetrodototoxin to show that these atropine resistant contractions have a nervous origin.

CONCLUSIONS

These data imply the involvement of the P2X$_1$ subtype. However, since only P2X$_2$ expression is increased in idiopathic detrusor instability bladders, it strongly implies that a P2X$_{1/2}$ heteromultimer is critical for the changes in purinergic innervation in idiopathic detrusor instability bladders. Our results support the myogenic theory of idiopathic detrusor instability, in that we have observed significant smooth muscle changes in idiopathic detrusor instability bladders by the quantification of calponin and smoothelin as well as by the decreased contractility of idiopathic detrusor instability samples. However, we also noted changes in P2X receptor quantity, specifically the up-regulation of P2X$_2$, differences in nerve mediated contractility and its enhanced adenosine triphosphate dependent component in idiopathic detrusor instability bladders, which supports the neurogenic hypothesis. These findings indicate that these mechanisms may contribute in a combined manner to the clinical manifestation of overactive bladder. Therefore, we conclude that these data indicate that purinergic innervation to the bladder is a novel target for the pharmacological manipulation of overactive bladder.

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