Loss of Purinergic $P2X_3$ and $P2X_5$ Receptor Innervation in Human Detrusor from Adults with Urge Incontinence

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Activation of purinergic P2X receptors associated with the parasympathetic nerves that supply the human bladder smooth muscle (detrusor) is implicated in control of detrusor contractility. The relative abundance of all seven subtypes colocalized with synaptic vesicles on parasympathetic nerves was examined in specimens from normal adult bladder, infants, and in adults with overactive detrusor contractility and a diagnosis of idiopathic detrusor instability (IDI) to determine whether receptor distribution varied with age or in patients with incontinence. Alteration in control of detrusor innervation was examined with P2X subtype-specific antibodies and an antibody against synaptic vesicles, using immunofluorescence and confocal microscopy. Detrusor samples were taken from: controls, at cystectomy for cancer or cystoscopic biopsy for hematuria ($n = 22$; age 33–88), child bladder, at surgical correction of vesicoureteric reflux ($n = 21$; age 4 months to 2 years), and adults with detrusor instability at cystoscopy–cystodistension ($n = 18$; age 30–81). Adult specimens contained muscle with large varicosities (1.2 μm) along parasympathetic nerves with colocalized patches of all $P2X_{1-7}$ subtypes. Infant bladder revealed little evidence of P2X at age <9 months but approached adult levels at 2 years. Detrusor from IDI patients revealed selective absence of $P2X_3$ and $P2X_5$ beneath all the varicosities. This specific lack of $P2X_3$ and $P2X_5$ may impair control of detrusor contractility and contribute to the pathophysiology of urge incontinence.

Key words: purinergic P2X receptors; hypertonia; human urinary incontinence; detrusor instability; innervation; IDI bladder

In the last two decades, the innervation of the smooth muscle of the human bladder (the detrusor) has received considerable attention, because increased detrusor muscle contractility is associated with urge incontinence (also known as detrusor instability). This type of urinary incontinence affects men and women across the life-span (Bower et al., 1996; Hunskar et al., 2000) and affects 25–40% of all those who seek help for incontinence (Moore, 1999), or ~5–20% of the population. Despite recent efforts, the pathophysiology of detrusor instability remains incompletely understood.

The efferent limb of the human micturition reflex is predominantly governed by the muscarinic receptor, which mediates detrusor contractility, with a minor contribution from adrenergic receptors in facilitating bladder relaxation. Increasing attention has also been paid to the subepithelial innervation (Moore et al., 1992) and the role of sensory neuropeptides in regulating afferent input (Smet et al., 1997), because patients with urge incontinence also experience a frequent strong need to micturate, both when awake and when asleep (nocturia), in association with a small bladder capacity.

Studies from lower-order mammals and from human detrusor have suggested that purines such as ATP may also be important in regulating detrusor contractility (Burnstock et al., 1978; Brown et al., 1979; Brading and Inoue, 1991; Bolego et al., 1995; Theobald, 1995). Changes in P2X receptor distribution that occur in pregnant, adult, and neonatal rats have been characterized (Hansen et al., 1998; Dutton et al., 1999; Yunaev et al., 2000). Recently, knock-out mice that lack the $P2X_3$ subtype were found to have markedly enlarged bladder capacity and reduced frequency of micturition (Cockayne et al., 2000). To date, the distribution of purinergic receptor subtypes has not been characterized in humans who have an increased frequency of micturition with a small-capacity, overly contractile bladder, nor has the morphology of P2X receptors been characterized in children of varying ages.

The aim of the present study was to examine human detrusor taken from control adults, neonates, infants, and adults with detrusor instability, to search for alterations in P2X receptor subtypes that may vary with age and/or have a bearing on the etiology of urge incontinence.

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MATERIALS AND METHODS

Tissue. Control detrusor samples were taken at cystectomy for nonirradiated bladder cancer or at cystoscopy for surveillance of previous low-grade malignancy or investigation of hematuria. Cystectomy specimens were taken from macroscopically normal areas of bladder. Biopsies were taken from the bladder base just lateral to the trigone in view of its distinct innervation pattern (Gosling and Dixon, 1975). Infant bladder specimens were taken at cystotomy during surgical correction of vesicoureteric reflux that was detected by antenatal ultrasonography with neonatal follow-up, or by sibling tracing. All subjects demonstrated radiological grade III-IV reflux (Lebowitz et al., 1985) and had failed to resolve spontaneously with antibiotic prophylaxis, as previously reported (Werkstrom et al., 2000); urine culture was sterile before surgery. Cystoscopic biopsies were taken from patients with proven idiopathic detrusor instability (as per urodynamic testing) who had failed to respond to antimuscarinic drugs for >12 months (Moore et al., 1992). Diagnostic urodynamic features (Abrams et al., 1990) were spontaneous detrusor contractions provoking an urgent desire to micturate during filling with warm sterile water, which the patient was unable to inhibit, accompanied by either an early first desire to void (<250 ml) or a reduced bladder capacity (<450 ml). Detrusor instability was characterized as idiopathic by virtue of the patients having no neurologic abnormality and no obstructive features, i.e., normal flow rate with no evidence of residual urine (Abrams et al., 1990). Because of failure to respond to standard anti-muscarinic therapy, patients underwent cystoscopy at which standard cold cup biopsy was taken for routine pathological examination to exclude carcinoma *in situ* that might account for refractory irritative symptoms. A deeper sample was then taken from the side wall of the small crater created by the first specimen, providing tissue 3 × 4 mm that contained detrusor muscle. All tissue collection was undertaken after informed consent in accordance with protocols approved by the local hospital ethical committee.

Materials. Antibodies specific to the extracellular domains of individual human P2X receptor subunits were produced in rabbits using similar epitopes to those used in the rat specific antibodies, as previously reported (Dutton et al., 1999). The small and nonhomologous sequences 68–84 ($P2X_1$), 209–226 ($P2X_2$) 185–303 ($P2X_3$), 270–285 ($P2X_4$), 272–288 ($P2X_5$), 200–218 ($P2X_6$), and 65–81 ($P2X_7$) were used with those from $P2X_1$ and $P2X_7$ each having an N-terminal Cys added for conjugation via diphtheria toxin using maleimidocaproyl-*N*-hydroxysuccinimide (Dutton et al., 1999). No cross-reactivity between subtypes was encountered when cRNA from the particular receptor was transfected into human embryonic kidney 293 cells and *Xenopus* oocytes. Further standard testing for specificity with adsorption controls showed that binding of each antibody was blocked in the presence of 10 μM of the individual cognate blocking peptide. SV2 monoclonal antibody was specific for the synaptic vesicle proteoglycan SV2 (Dutton et al., 1999). Cyanine2 and Cyanine5 conjugates of donkey anti-rabbit and donkey anti-mouse fluorescent secondary antibodies, adsorbed against conspecific IgGs were purchased from Jackson ImmunoResearch (West Grove, PA). All other reagents were purchased from Sigma (St. Louis, MO).

Immunohistochemical methods. Human bladder tissue was fixed in 4% paraformaldehyde in PBS buffer, pH 7.2, for 6 hr. The tissue was then cryoprotected by immersion in 30% sucrose for 24 hr before sections (30 μm) were cut on a freezing microtome, and sections were labeled as described (Hansen et al., 1998; Dutton et al., 1999; Yao et al., 2000; Yunaev et al., 2000). Three tissue sections from each patient, including at least 10 high-power fields from each section, were viewed on a Leica (Nussloch, Germany) TCS NT UV laser confocal microscope system, with the pinhole set at 1.0 as a compromise between focal depth and background fluorescence. The monoclonal antibody to the proteoglycan SV2 was used to immunolocalize nerve varicosities. These were only rarely able to be labeled with an antibody to tyrosine hydroxylase and thus are identified as being the parasympathetic nerves in the body of the bladder detrusor, rather than sympathetic (Theobald, 1995). SV2 immunoreactivity manifested as spheroidal puncta of ~1.2 μm in diameter. The varicosities, labeled with SV2 and the mouse Cy5 secary were then used as reference points to determine the relationship of the labeled P2X receptors to the parasympathetic detrusor nerves. By using confocal microscopy, each P2X receptor subtype that was labeled with the rabbit Cy2 secary and was colocalized with a varicosity was counted individually. Controls in which only one primary and/or one secondary antibody was used revealed no breakthrough of fluorescence between the two widely separated channels at 525 nm (Cy2) and 665 nm (Cy5). Not all SV2/Cy5-labeled varicosities were labeled with a corresponding P2X/Cy2 antibody. Each SV2/Cy5-labeled varicosity in each field of view was counted, and the corresponding varicosities labeled with each of the P2X/Cy2 labels was then recorded from the separate channel, and the number of coincident labels was tabulated. Relative intensities of the different P2X labels were compared with the intensity of the SV2 labels from different slides, and results were quantitated using NIH Image software. Comparisons between populations of specific receptor types from adult controls and IDI tissue were made using the unpaired *t* test with two-tailed *p* values obtained. Values of $p < 0.05$ were considered significant.

RESULTS

P2X receptors in neonates, infants, and control adults

All seven P2X receptor subunits ($P2X_{1-7}$) exhibited specific immunoreactivity in older children (>2 years) and adults. Large P2X puncta, ~1.2 μm in diameter were found closely appositioned to presynaptic vesicles labeled with SV2. In young infants of <9 months, no P2X receptor labeling was found in relation to the clearly apparent strings of varicosities on the nerves. Figure 1*A* shows an abundance of clearly resolved varicosities in strings outlining nerves in the detrusor from an 8-month-old infant. None of these varicosities were colocalized with $P2X_2$ (Fig. 1*B*) or indeed any other P2X subtype. These are shown at higher resolution in Figure 1, *C* and *D*.

At 2 years, most infants exhibited clearly colocalized P2X subtypes adjacent to the SV2-labeled varicosities. A representative example is shown of SV2 and $P2X_3$ in Figure 1, *E* and *F*. It should however be noted that the relative abundance of the P2X receptor subtypes found colocalized with the varicosities was low. The size of the SV2 puncta were generally much larger than the corresponding size of the P2X puncta, indicating that the varicosities were not completely apposed with P2X receptor at this age.

In control adult bladder, the varicosities that are identified by SV2 labeling are routinely colocalized with $P2X_{1-3}$ and $P2X_5$ but the abundances of $P2X_4$, $P2X_6$ and especially $P2X_7$ are much lower, with many varicosities appearing entirely devoid of these receptors with other varicosities exhibiting sparse receptor labeling. A representative example labeled with $P2X_5$ is shown in Figure 1, *G* and *H*, in which SV2-labeled varicosities exhibit an abundance of colocalized $P2X_3$ receptor. The size of the P2X puncta in adult tissue is commensurate with the size of the SV2 puncta, indicating a more extensive association of the P2X receptor patches with the varicosities on the parasympathetic nerves.

Table 1 summarizes results of measurements of varicosity colocalization with the P2X subtypes in the different tissues. A total of eight young infants aged 4–9 months, eight infants aged 10–18 months, and five children aged 2 years were examined together with 22 adult controls and 18 IDI patients. In the case of the young infants aged 4–9 months, no P2X subtypes were found colocalized with the varicosities on the nerves in the detrusor. In the 10–18 month age group, individual labeling of varicosities with P2X subtypes commenced, but levels were quite variable, and thus averages in this category have not been presented. By 2 years, the degree of colocalization had reached an equilibrium with the majority of varicosities appearing to be labeled with all subtypes of P2X receptor, with only $P2X_4$ and $P2X_6$ not being associated with all varicosities. The size of the P2X puncta was still somewhat smaller than the size of puncta from adult tissue.

Alterations of P2X distribution in adults with urge incontinence

The results in 22 adult control bladders (8 female and 14 male) tested revealed a consistent pattern of receptor colocalization.

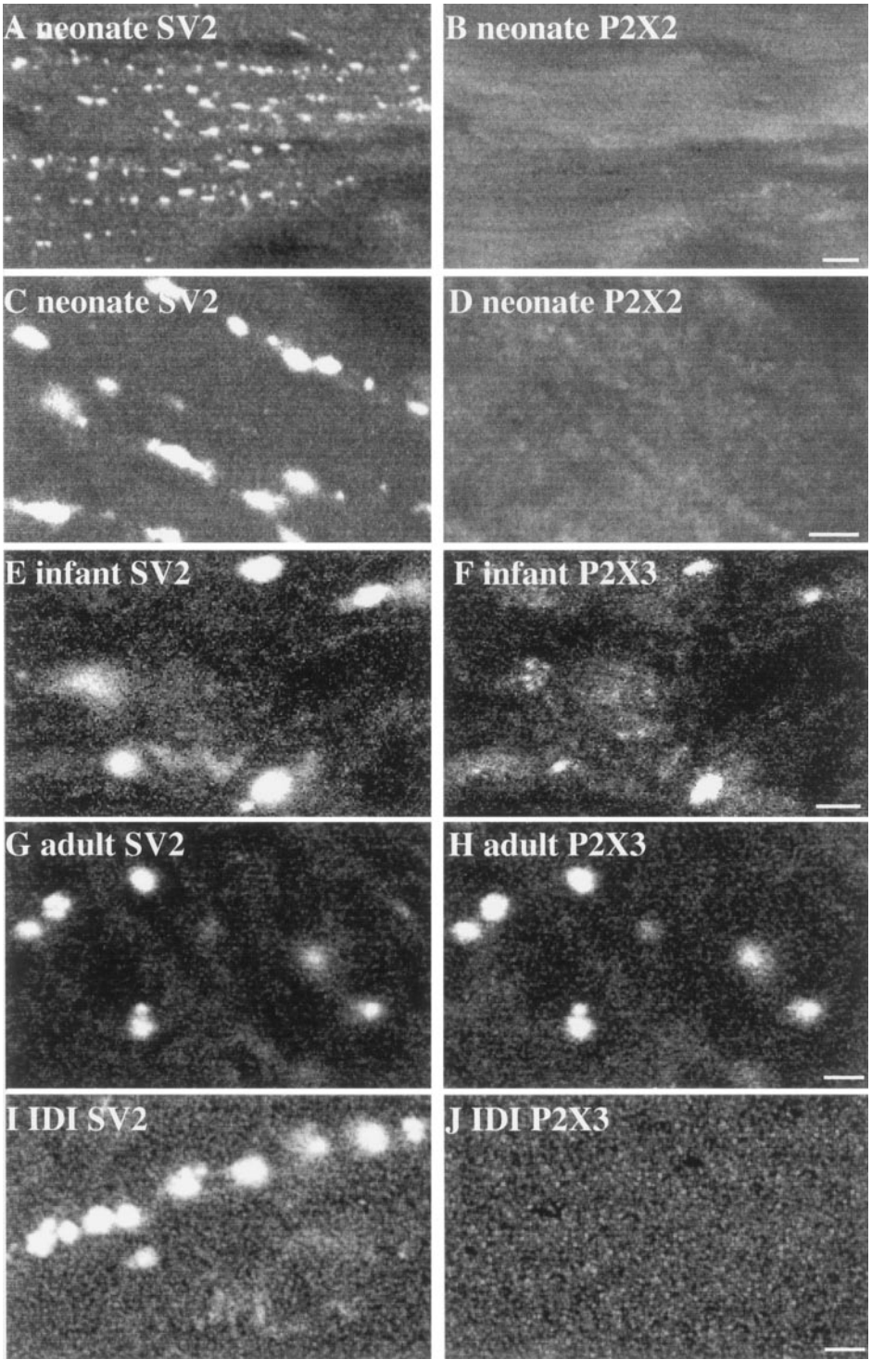

Figure 1. *A* and *B* show a representative image pair of bladder detrusor muscle from an 8-month-old labeled for SV2 and $P2X_2$, respectively. The vesicles in the long strings of varicosities on the parasympathetic nerves are labeled, but there is no corresponding $P2X_2$ label, nor is there any other P2X subtype present at this age. Scale bar, 5 μm. *C* and *D* show an enlargement of *A* and *B*. Scale bar, 2 μm. *E* and *F* show detrusor taken from a 2-year-old labeled for SV2 and $P2X_3$, respectively. Like other subtypes, the $P2X_3$ is found colocalized with the large varicosities to various extent. Scale bar, 2 μm. *G* and *H* show a string of varicosities from adult control bladder labeled with $P2X_3$. This is representative of subtypes $P2X_1$, $P2X_2$, and $P2X_5$ with other subtypes appearing at much lower levels in the adult. Scale bar, 1 μm. *I* and *J* show an example taken from a patient with IDI labeled with SV2 and $P2X_3$. Like $P2X_5$, $P2X_3$ is completely downregulated in these patients, whereas other subtypes remain essentially unaltered. Scale bar, 2 μm. SV2 primary antibody was labeled with Cy5 secary, whereas all P2X antibodies were labeled with Cy2 secary.

Tissue samples collected from either males or females at cystoscopy or cystectomy exhibited similar patterns of colocalization between P2X receptors and the SV2-labeled varicosities. Almost all varicosities were colocalized with $P2X_{1-3}$ and $P2X_5$. In contrast, very few varicosities were observed to be colocalized with $P2X_4$, $P2X_6$, and $P2X_7$ receptor subtypes. When present at all, the intensity of receptor labeling on these varicosities appeared to be much lower than $P2X_{1,2,3,5}$ (Table 1).

The 18 patients with IDI were women aged 30–81 years. Urodynamic testing of these patients revealed the first desire to void occurred at an average 173 ml (range, 50–340 ml), and the average maximum bladder capacity was 340 ml (range, 150–570 ml). The average maximum detrusor pressure was 48 cm H_2O (range, 18–100 cm H_2O). At microscopy, we were unable to observe any SV2-labeled varicosities that were colocalized with either of the subtypes $P2X_3$ or $P2X_5$ (Fig. 1*I*,*J*). The expression or synthesis of these two subtypes appeared markedly reduced in the detrusor from IDI patients. In the unstable muscle, $P2X_4$ and $P2X_6$ subtypes were more commonly associated with SV2-staining varicosities than in control bladders (36 and 33% vs 16

Table 1. The percentage of colocalization of SV2-labeled parasympathetic nerve varicosities in the body of the detrusor with P2X subtypes in the groups 4–9 months, 2 years, control, adult, and IDI

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
Neonate (8)	0/230	0/243	0/222	0/219	0/238	0/229	0/208
4–9 month	0	0	0	0	0	0	0
Infant (5)	146/153	141/143	109/110	145/201	193/210	93/223	121/140
2 year	95	99	99	72	92	42	86
Adult (22)	806/827	836/844	763/809	139/892	715/783	156/892	36/591
33–88 year	97	99	94	16	91	18	6
IDI (18)	518/538	548/556	0/555	194/532	0/511	178/544	156/232
30–81 year	96	99	0	36	0	33	67
Adult/IDI	$p = 0.32$	$p = 0.16$		$p < 0.000$		$p < 0.0001$	$p < 0.0001$

Twenty to forty SV2-labeled varicosities were counted for each P2X receptor subtype for each patient sample. Immunofluorescent intensity of $P2X_4$, $P2X_6$, and $P2X_7$ in adult and IDI were all at least 10-fold lower than other subtypes such as $P2X_1$, as determined using NIH Image analysis, indicative of much lower expression levels of these receptors. The extreme differences between $P2X_3$ and $P2X_5$ expression in normal adult and IDI patients are clearly evident. Comparison between $P2X_{4,6,7}$ expression in these two cohorts also showed highly significant differences ($p < 0.0001$; unpaired two-tailed t test).

and 18%, respectively), but like the control bladders, image analysis showed that the intensity of the Cy2 fluorescence with these subtypes was low compared with $P2X_1$ and $P2X_2$ (<10%). The majority of SV2-labeled varicosities from IDI patients were immunolocalized with trace amounts of $P2X_7$, whereas control bladder exhibited far fewer varicosities with any detectable $P2X_7$ immunofluorescence, although these were brighter. The levels observed were typically <10% of the levels observed in varicosities colocalized with $P2X_1$ and $P2X_2$.

DISCUSSION

Immunohistochemical studies have demonstrated the existence of $P2X_{1-6}$ subtypes in rat bladder detrusor (Hansen et al., 1998; Dutton et al., 1999; Yunaev et al., 2000) but, until recently, evidence for the existence of P2X receptors in human bladder has been limited (Bo and Burnstock, 1995; Evans et al., 1996; Longhurst et al., 1996; Bayliss et al., 1999).

The adult rat bladder detrusor receives a dense innervation from parasympathetic nerve terminals (Hoyes et al., 1975) but very little sympathetic innervation, with most of this restricted to the trigone (Gosling and Dixon, 1975). During development of the rat bladder, the micturition reflex, including a mature spinobulbospinal element, is not established before 2–3 postnatal weeks (Araki and de Groat, 1997). During this time there is considerable increase in the atropine-resistant component of contraction of the detrusor in response to parasympathetic nerve stimulation (Maggi et al., 1984), which has recently been shown to be caused by purinergic transmission (Bayliss et al., 1999). It is during this period of development that P2X receptors become established beneath varicosities in the rat detrusor muscle, providing for an increase in the extent of purinergic transmission to the smooth muscle cells (Dutton et al., 1999). The rich purinergic supply to the urinary bladder found in many other species, including human, suggests that purinergic transmission may be involved in initiating contraction and urine flow from the bladder (Theobald, 1995). The response of the bladder in many species such as guinea pig to single intramural nerve impulses however is biphasic, with the fast phasic contraction caused by ATP followed by a slower tonic contraction induced by acetylcholine (Brading and Inoue, 1991). The relative contributions of these two phases of the response to single pulses differs between species, but approximately half the contractile response can be attributed to purinergic transmission and the remainder to cholinergic transmission (Levin et al., 1991). The latter is probably responsible for the maintenance of bladder contraction and urine flow after this has been initiated by purinergic transmission (Theobald, 1995). In humans however, the purinergic control is less clear (Inoue and Brading, 1991). Normal human bladder strips were found to elicit very little purinergic nerve-mediated response, although direct application of ATP agonist elicited very large responses. It has been suggested that the closenesss of innervation and extent of cell–cell coupling in humans may explain these results (Inoue and Brading, 1991). In contrast, strips taken from IDI bladders did show direct purinergic responses to stimulation of the intrinsic nerves (Bayliss et al., 1999).

In this study we have established that P2X receptor subtypes found subsynaptically in rat (Dutton et al., 1999) are similarly found to be closely associated with the parasympathetic varicosities in human detrusor muscle and that, like the young rat pup bladder, infants of <10 months appear to lack purinergic innervation and thus lack effective bladder control. Only after 2 years of age are the varicosities consistently colocalized with the P2X receptors. It is only at this stage of child development that more effective bladder control becomes established, and this can vary with the individual. It should be noted that the proportion of varicosities colocalized with the subtypes $P2X_4$, $P2X_6$, and $P2X_7$ in the 2 year olds (Table 1) is similar to the levels in the IDI patients in that they are localized under more varicosities than in normal adults, and this may suggest a similar immature control of contractility in the IDI bladders. By adulthood, the normal pattern of expression is closely similar to that we have previously found in adult rats (Dutton et al., 1999; Yunaev et al., 2000). This consistent pattern of expression provides the basis for an examination of the role of P2X receptors in the pathophysiology of dysfunctional bladders in humans.

The identification of subsynaptic P2X receptors in normal bladder is consistent with observations that normal and idiopathic unstable human detrusor contracts in response to ATP (Tagliani et al., 1997; Bayliss et al., 1999). However, any additional purinergic component in the unstable detrusor appears not to be attributable to stimulation of extrajunctional receptors that may be more accessible to ATP from disrupted nerves because subjunctional receptors are found in both tissues.

The question arises why IDI bladders exhibit a purinergic current from direct nerve stimulation whereas normal bladders apparently do not. Of particular interest is the altered pattern of expression of receptors in the condition IDI, with $P2X_3$ and $P2X_5$

no longer being observed beneath the varicosities. Cystometry studies in the $P2X_3$ knock-out mouse (Cockayne et al., 2000) revealed that a marked increase in bladder capacity occurred in the absence of $P2X_3$. Because the converse is found in IDI patients, i.e., all displayed marked urge incontinence, with reduced bladder capacity, we expect that $P2X_5$ is similarly essential for full control of the micturition initiation signal, if not through direct nerve stimulation, then certainly through an alteration in cell–cell coupling in the detrusor muscle. Further studies of the micturition reflex in the $P2X_5$ knock-out mouse will be needed to confirm this hypothesis. Nevertheless, the striking absence of $P2X_3$ and $P2X_5$ labeling in relation to parasympathetic nerve varicosities that we observed for the first time in patients with urge incontinence (in sharp contrast with the pattern seen in control adult specimens and the older infants) suggests that the absence of these two receptor subtypes is related to the pathophysiology of detrusor instability. Partial loss of purinergic control observed in IDI may cause loss of inhibition of micturition initiation signals. This may manifest as a loss of inhibition of acetylcholine release at the varicosities. Certainly young infants lacking P2X receptors have no effective bladder control, so the urge resulting from progressive bladder filling cannot be suppressed. Subsynaptic P2X receptors may fulfil this role. In serial examination of the superior cervical ganglia of the rat pup, there is a progressively greater appearance of P2X receptors at this location (Li et al., 2000) and in peripheral sites such as bladder sequentially after day 1 (Dutton et al., 1999). Thus, with increasing maturity, there is increasing central and peripheral evidence of P2X distribution in the rat pup. Other observations indicate that P2X receptors are progressively delivered to the parasympathetic nerves of the bladder, with $P2X_2$ being the first subtype to arrive in the axons in the detrusor of day 1 rats with others like $P2X_3$ also arriving postsynaptically (Dutton et al., 1999).

Adults with urge incontinence often have difficulty focusing the frontal lobe of their cerebral cortex on the inhibition of the desire to void. This activity, called "bladder training" is an essential part of continence treatment for patients with IDI. The process requires them to ignore afferent stimuli from progressive bladder filling. Theoretically, adults with IDI may be suffering from a lack of purinergic receptor maturity in the periphery, perhaps in association with poor coordination and integration of the incoming stimuli at the locus of the cerebral cortex. Thus, there may be a mismatch between the normal inhibitory actions of the $P2X_3$ and/or $P2X_5$ receptors and the excitatory effects of the other P2X subtypes. Very early observations of the purinergic innervation of the subepithelial layers of patients with IDI indicate that both these subtypes are present in the lamina propria, suggesting a selective deficit in the detrusor, but further collection of subepithelial specimens is awaited. The overall mechanism may be that the purinergic inhibitory control of the parasympathetic release of acetylcholine is disrupted in IDI.

Aside from the total loss of expression of these two normally abundant subtypes in IDI, the minor subtypes $P2X_4$, $P2X_6$, and $P2X_7$ all exhibit increased subsynaptic distribution ($p < 0.0001$), albeit at lower densities than found in normal adult tissue. It may be a combination of the total loss of the rapid desensitizing subtypes $P2X_3$ and $P2X_5$ that are expected to internalize in response to ATP application (Li et al., 2000) in combination with a small increase in overall distribution of the nondesensitizing subtypes $P2X_4$ and $P2X_6$ that leads to an overall prolongation of purinergic response seen in the IDI detrusor after application of agonist. Thus, previous emphasis on research into new antimuscarinic agents for the treatment of urge incontinence may now be modified to encourage a search for agents that affect regulation of the purinergic (P2X) system in the human detrusor.

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