

Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis

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Birder, L. A., H. Z. Ruan, B. Chopra, Z. Xiang, S. Barrick, C. A. Buffington, J. R. Roppolo, A. P. D. W. Ford, W. C. de Groat, and G. Burnstock. Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. *Am J Physiol Renal Physiol* 287: F1084–F1091, 2004. First published July 13, 2004; doi:10.1152/ajprenal.00118.2004.—Purinergic mechanisms appear to be involved in motor as well as sensory functions in the urinary bladder. ATP released from efferent nerves excites bladder smooth muscle, whereas ATP released from urothelial cells can activate afferent nerves and urothelial cells. In the present study, we used immunohistochemical techniques to examine the distribution of purinoceptors in the urothelium, smooth muscle, and nerves of the normal cat urinary bladder as well as possible changes in the expression of these receptors in cats with a chronic painful bladder condition termed feline interstitial cystitis (FIC) in which ATP release from the urothelium is increased. In normal cats, a range of P2X (P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, P2X₆, and P2X₇) and P2Y (P2Y₁, P2Y₂, and P2Y₄) receptor subtypes was expressed throughout the bladder urothelium. In FIC cats, there is a marked reduction in P2X₁, and loss of P2Y₂ receptor staining. Both P2X₃ and P2Y₄ are present in nerves in normal cat bladder, and no obvious differences in staining were detected in FIC. Smooth muscle in the normal bladder did not exhibit P2Y receptor staining but did exhibit P2X (P2X₂, P2X₁) staining. In the FIC bladder smooth muscle, there was a significant reduction in P2X₁ expression. These findings raise the possibility that purinergic mechanisms in the urothelium and bladder smooth muscle are altered in FIC cats. Because the urothelial cells appear to have a sensory function in the bladder, it is possible that the plasticity in urothelial purinergic receptors is linked with the painful bladder symptoms in IC.

feline interstitial cystitis; ATP; urothelium

INTERSTITIAL CYSTITIS (IC) is a chronic pelvic pain syndrome of unknown cause and no generally accepted treatment (32). Symptoms include pain referable to the urinary bladder and increased frequency and urgency of urination. A comparable disorder occurring in domestic cats termed feline interstitial cystitis (FIC) exhibits many of the hallmarks of human IC (5). Although recent studies suggest the involvement of the urothelium in this disorder, the etiology is still unknown.

Some studies have revealed that the urothelium is “leaky” in IC cats and humans, which allows noxious substances to stimulate bladder afferents (14, 17). Studies have also lent support for the idea that mechanical stimuli can evoke the

release of ATP from epithelial cells lining “tubes” or “sacs” such as the urinary bladder (9, 39) and that extracellular ATP, most likely of urothelial origin, is implicated in the distension-evoked activation of bladder afferents (7). ATP released from the urothelium is thought to activate P2X_{2/3} receptors in afferent nerves adjacent to the urothelium and plays a role in bladder function (30). In fact, mice lacking the P2X₃ purinergic receptor subunit (normally expressed by a subset of bladder afferents) exhibit normal distension-evoked urothelial ATP release but diminished reflex bladder contractions and voiding behavior as well as a reduction in the behavioral (pain) response to injection of ATP (10, 39). Thus mechanically evoked ATP release from epithelial cells may play a major role in both volume- and pain-mediated responses.

In addition, urothelial cells isolated from patients as well as cats diagnosed with IC release significantly larger amounts of ATP than normal cells following stretch stimuli (1, 33). Increased release of ATP has also been demonstrated following tissue injury or inflammation (3). In the bladder, altered release of ATP from either injured or sensitized urothelial cells may play a direct role in sensitizing nociceptors underlying the urothelium and thereby contribute to the initiation of pain and inflammatory responses.

Multiple P2 purinergic receptor subtypes have been located in a number of epithelial tissues, on both apical as well as basolateral surfaces (18, 21, 34). It has been suggested that activation of P2 receptors in epithelia may elicit various effects including secretion and vesicle trafficking as well as transmitter release (16, 19). Bladder biopsy specimens from adults with bladder obstruction or detrusor instability revealed that the distribution of purinergic receptors could be altered in pathological conditions (20, 23, 24). It remains to be determined whether similar alterations are present in IC.

The distribution of P2 receptors has been previously characterized in normal rodent and human bladder tissue (23, 24, 28). The aim of the present study was to examine urinary bladders from normal cats and those diagnosed with FIC to investigate whether an alteration in P2 receptor subtype expression may have a role in the etiology of IC.

MATERIALS AND METHODS

All procedures were conducted in accordance with Institutional Animal Care and Use Committee policies.

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Animals. Healthy and FIC adult cats were used for this study. All cats with FIC were obtained as donations from clients due to a history of chronic recurrent stranguria, hematuria, pollakiuria, and/or urinations in inappropriate locations. Evaluation at the Ohio State University (OSU) Veterinary Teaching Hospital consisted of a complete physical examination (including body weight), complete blood count, serum biochemical analysis, urinalysis, urine bacteriologic culture, and cystoscopy. Cystoscopy was performed using a 9-F rigid pediatric cystoscope (Karl Storz, Endoscopy America, Culver City, CA) in female cats and a 3-F flexible fiber optic cystoscope (Five Star Medical, San Jose, CA) in the male cats. The diagnosis of FIC was based on compatible history and consideration of standard National Institutes of Health inclusion and exclusion criteria as well as the results of the above laboratory tests, including the presence of submucosal petechial hemorrhages (glomerulations) at cystoscopy. Healthy, age-matched cats obtained from commercial vendors and determined to be free of disease and signs referable to the lower urinary tract according to the same diagnostic criteria as cats with FIC were used as controls. All cats were housed in stainless steel cages in the OSU animal facilities and allowed to acclimate to their environment for at least 3 mo before study.

Tissue and materials. Urinary bladders were excised from deeply anesthetized (α -chloralose 60–70 mg/kg; 2% halothane) cats (of either sex). After removal of tissue, the animals were killed via overdose of anesthetic. Anesthesia was determined to be adequate for surgery by periodically testing for the absence of a withdrawal reflex to a strong pinch of a hind paw and absence of an eye blink reflex to tactile stimulation of the cornea. The preparation and characterization of the primary rabbit polyclonal antisera to purinergic receptors used in this study have been previously reported (20) P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, P2X₆, P2X₇ (Roche Palo Alto, Palo Alto, CA) and P2Y₁, P2Y₂, P2Y₄ (Alomone Labs, Jerusalem, Israel). No cross-reactivity between subtypes was detected and further testing for specificity showed that staining was eliminated when the primary antibody was eliminated or added in the presence of the specific homolog peptide antigen. Cyanine3 (Cy3) conjugates of donkey anti-rabbit fluorescent secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Immunofluorescence methods. The bladders were gently cut open and stretched (urothelial side up), postfixed in paraformaldehyde (4%) followed by sucrose (30%), and rapidly frozen by immersion in isopentane/dry ice (−70°C for 2 min). Tissue sections taken from bladder detrusor (matched between FIC and normal cats) were cryosectioned (10 μ m; minimum 4 sections from each whole bladder) and thaw-mounted on poly-L-lysine-coated slides. An indirect immunofluorescence method was used to visualize receptor expression. Briefly, sections were incubated overnight with the primary antibodies [3 μ g/ml with 10% normal goat serum (NGS) in PBS containing 0.05% merthiolate and 0.2% Triton X-100]. Subsequently, the sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (1:300 in 1% NGS in PBS containing 0.05% merthiolate for 1 h). Slides were mounted with Citifluor (UKC Chem Labs, Kent, UK) and examined with fluorescence microscopy.

To demonstrate the colocalization of either P2X₃ or P2Y₄ receptors with PGP 9.5, sections were incubated overnight with an antibody for one of the purinergic receptors and with a monoclonal PGP 9.5 antibody (Research Diagnostics, Flanders, NJ). The sections were then detected with Cy3-conjugated donkey anti-rabbit IgG or FITC-conjugated mouse antibodies. Images of immunofluorescence labeling were taken with a Leica DC 200 digital camera (Leica, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Germany). Images were imported into a graphics package and the two-channel readings for green and red fluorescence were merged (Adobe Photoshop 7.0).

RESULTS

Urinary bladder urothelium. In the urothelium from normal cat urinary bladder, expression of all seven P2X purinergic receptor subunits was detected throughout both the basal and apical layers. The predominant subtype was P2X₃ (based on staining intensity) followed by P2X₂, P2X₁, P2X₄, P2X₅, P2X₆, and P2X₇. A representative example is depicted in Fig. 1.

In addition, P2Y₁, P2Y₂, and P2Y₄ purinergic receptor subunits are also expressed throughout the urothelium in the normal cat bladder. In the normal bladder, there was no significant difference in staining throughout the epithelial layers nor was there any significant difference in either distribution or density of staining between cats.

Tissue samples collected from FIC cats showed a consistent pattern of purinergic receptor expression. In contrast to normal cats, there was a significant decrease in P2X₁ staining in FIC urothelium in all tissues sampled, with no significant difference in expression pattern for any of the other subtypes (Figs. 1 and 2). In addition, there was also a significant reduction of P2Y₂ expression in FIC compared with normal bladder (Figs. 2 and 3).

Bladder nerves. Nerve fibers in the subepithelial nerve plexus (labeled with PGP 9.5) show positive immunostaining for P2X₃ receptors (Fig. 4). P2X₃-positive nerve bundles (that colocalize with PGP 9.5) were also detected within the bladder smooth muscle (Fig. 4). In addition, P2Y₄ staining was also detected in nerve bundles (also colocalized with PGP 9.5) near the urothelium as well as in the bladder smooth muscle (Fig. 4). Although it has been suggested that nerves exhibiting P2X receptor staining are decreased in bladders from patients with various sensory dysfunctions (24), we were unable to observe any significant difference in density of P2X₃ or P2Y₄ staining within either the subepithelial nerve plexus or smooth muscle nerve bundles in the FIC cat.

Urinary bladder smooth muscle. P2X receptors in smooth muscle are thought to mediate bladder contractions. Thus we examined the distribution of various P2X receptor subunits in urinary bladder smooth muscle. P2X₂ is the predominant purinergic subtype within normal bladder smooth muscle with lower expression of P2X₁ followed by P2X₃ and P2X₇. P2X₄, P2X₅, and P2X₆ staining was not detected. In addition, we did not detect staining of P2Y receptors in bladder muscle. Compared with P2X₂ expression, P2X₁ staining in the smooth muscle was less in FIC than in normal cats (Fig. 5).

DISCUSSION

This study revealed multiple P2X and P2Y receptor subtype staining at various sites in the cat urinary bladder and differences in the intensity of staining in bladders from normal cats and cats with FIC. The most complex receptor expression occurred in the urothelium, which exhibited a range of P2X (P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, P2X₆, P2X₇) and P2Y receptors (P2Y₁, P2Y₂, and P2Y₄). Bladder smooth muscle also exhibited a range of P2X receptors but no P2Y receptors, whereas bladder nerves expressed only P2X₃ and P2Y₄ receptors. Based on the intensity of immunohistochemical staining, the dominant receptor subtypes were P2X₃ in urothelium, P2X₂ in smooth muscle, and P2X₃ in the nerves.

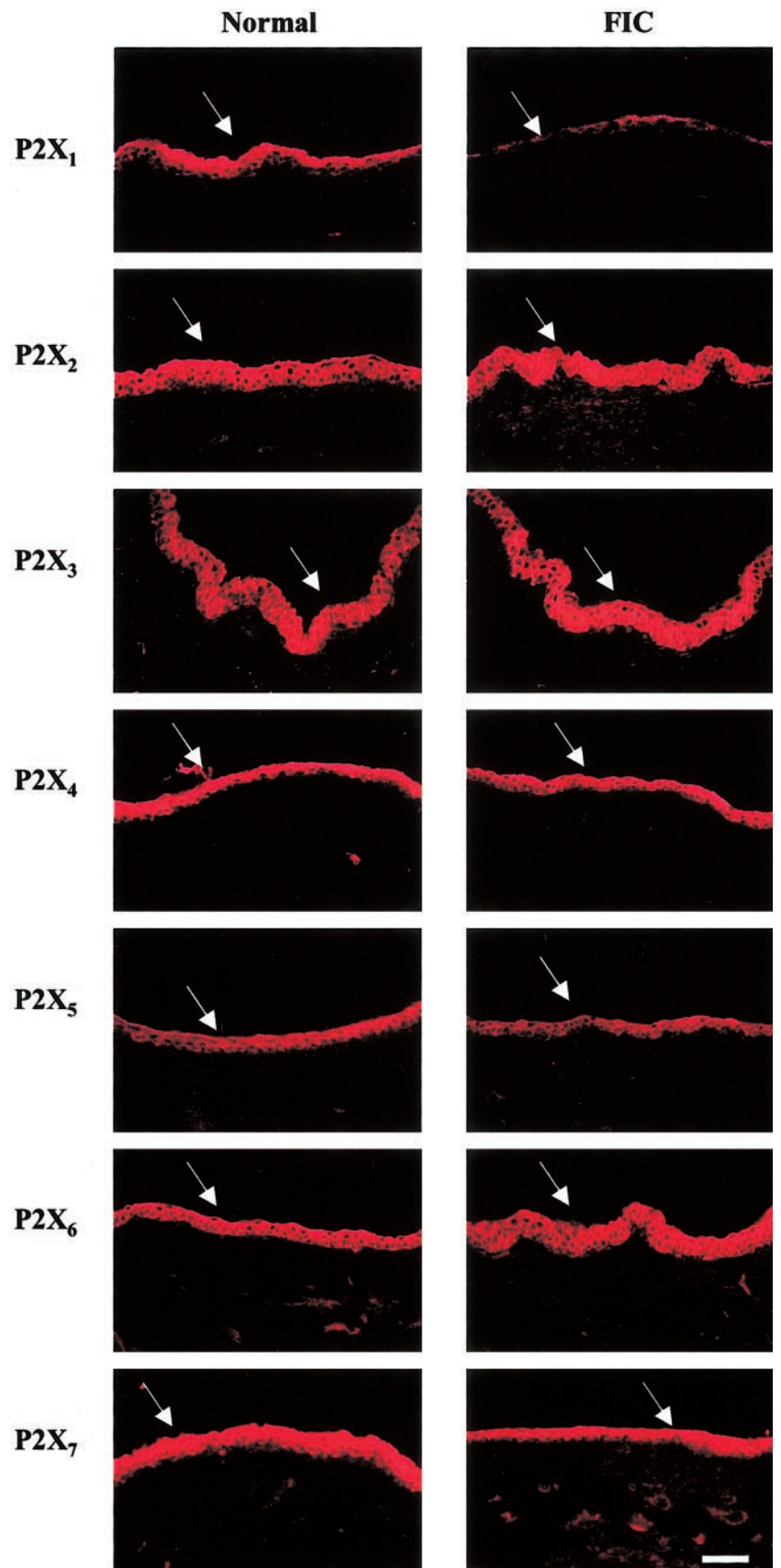


Fig. 1. Localization of P2X receptor (P2X₁₋₇) immunoreactivity in normal (*left*) and feline interstitial cystitis (FIC; *right*) cat urothelium. White arrows on representative images indicate mucosal (urothelial) surface. Scale bar = 50 μ m.

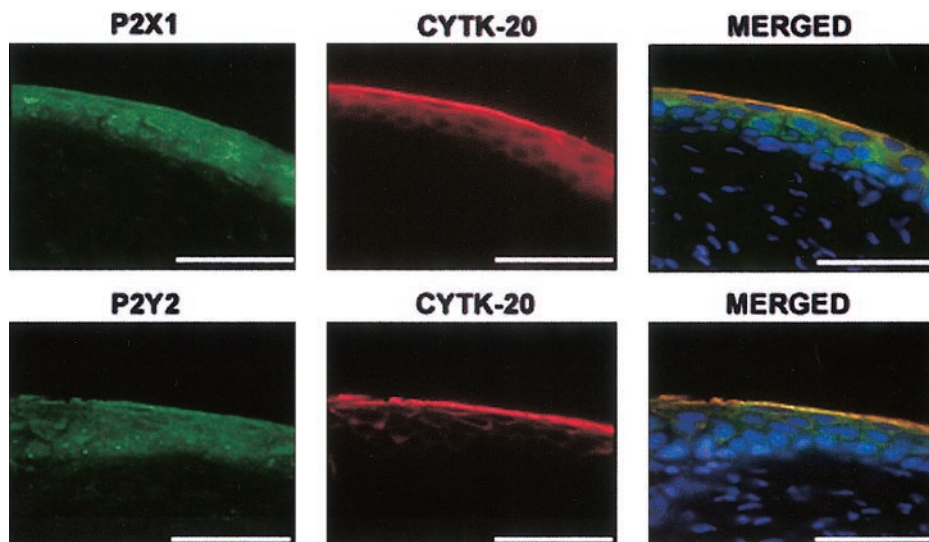


Fig. 2. Localization of P2X₁ and P2Y₂ immunoreactivity (IR) in normal cat urothelium. *Left*: P2X₁-IR or P2Y₂-IR throughout the urothelium. *Middle*: cyokeratin-20 (marker for apical urothelial cells). *Right*: representative merged images illustrating the colocalization of P2X₁ or P2Y₂ and cyokeratin as well as DAPI (nuclear marker) within the bladder urothelium. Scale bar = 100 μ m.

The urothelium showed the most obvious differences between normal and FIC cats (i.e., a decrease in P2X₁ and P2Y₂ receptor staining in FIC cats), which is consistent with the view that the functions of the urothelium are altered in IC. The urothelium, which has “neuron-like” properties including the ability to respond to and release ATP, has been implicated in sensory mechanisms in the urinary bladder (2, 15, 39). The present results raise the possibility that purinergic modulation

of the urothelial sensory functions as well as barrier functions might be altered in IC.

In bladder smooth muscle, activation of P2X receptors elicits excitatory effects mediated by membrane depolarization and influx of Ca²⁺ (22). Multiple P2X receptors have been identified in rat and human bladders; however, it is generally believed that P2X₁ receptors are the predominant subtype involved in the neurally evoked purinergic excitatory effects

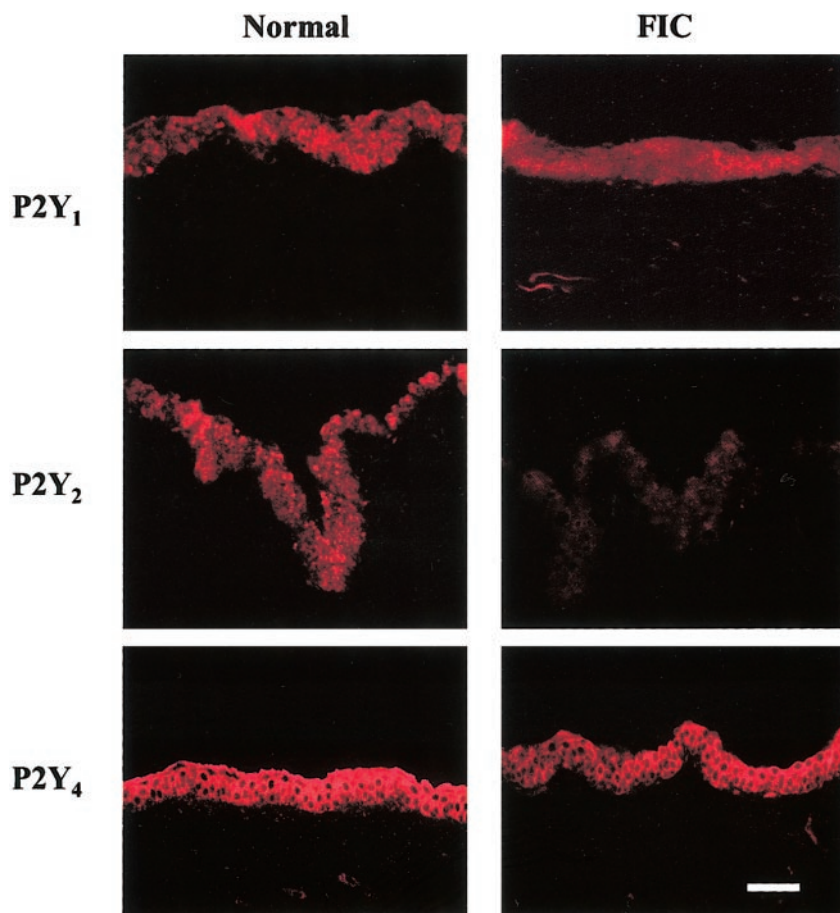


Fig. 3. Localization of P2Y receptor IR in normal (*left*) and FIC (*right*) cat urothelium. *Top*: mucosal surface and P2Y₁-IR. *Middle*: P2Y₂-IR. *Bottom*: P2Y₄-IR in urinary bladder urothelium. Scale bar = 50 μ m. Note decrease in P2Y₂ expression in FIC urothelium.

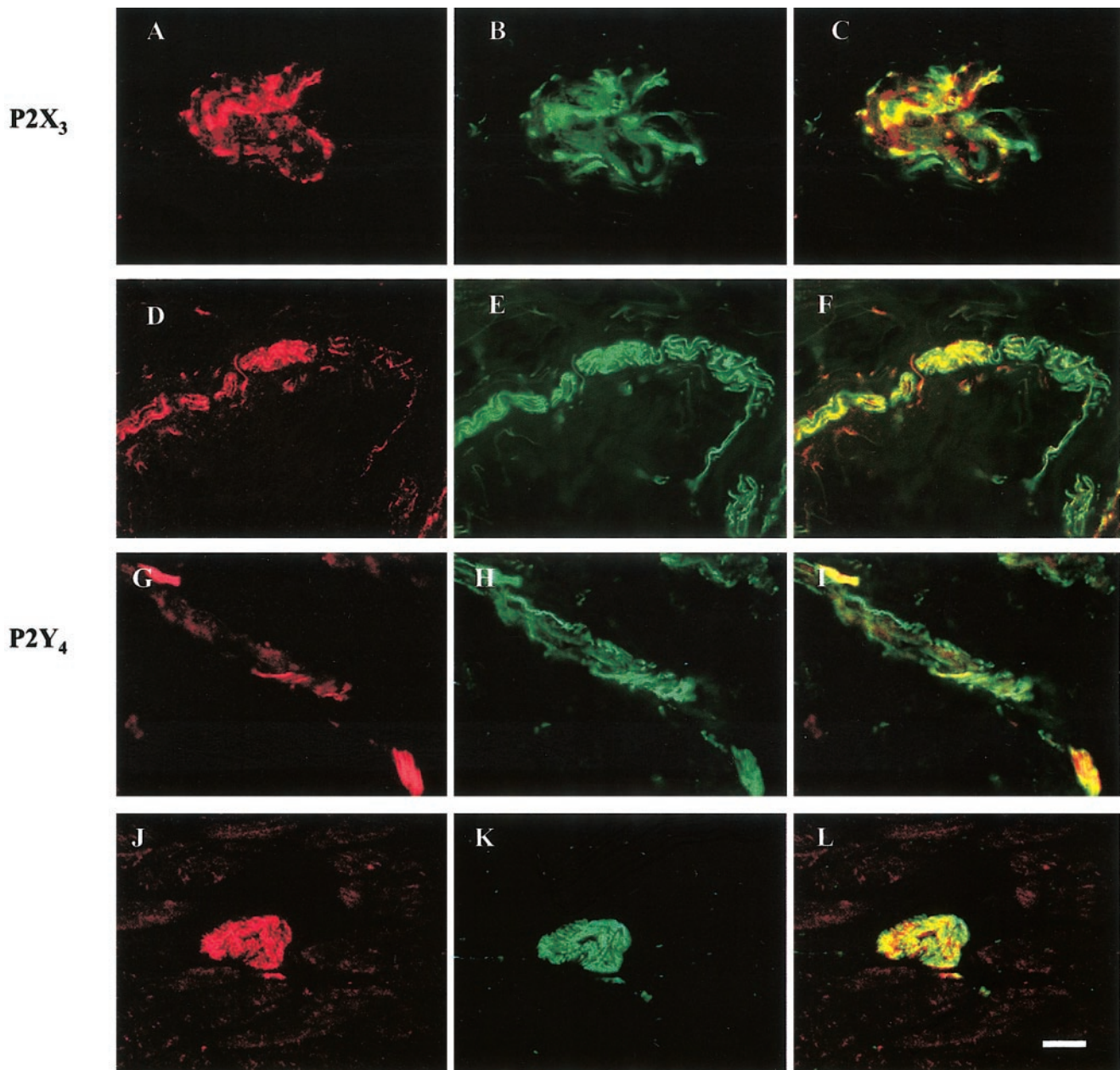


Fig. 4. Colocalization of P2X₃ or P2Y₄ receptors with PGP9.5 in cat bladder nerves within bladder submucosal plexus or bladder smooth muscle. A-C: image depicts P2X₃-IR nerve bundles (A, red) also stained for PGP9.5 (B, green). C: image depicts a representative merged image illustrating the colocalization of P2X₃ and PGP9.5 in the subepithelial nerve plexus. D-F: image depicts P2X₃-IR nerve fiber (A, red) also stained for PGP9.5 (B, green). C: image depicts a representative merged image illustrating the colocalization of P2X₃ and PGP9.5 in nerve bundles within the bladder smooth muscle. G-I: image depicts P2Y₄-IR nerve bundles (A, red) also stained for PGP9.5 (B, green). C: image depicts a representative merged image illustrating the colocalization of P2Y₄ and PGP9.5 in the subepithelial nerve plexus. J-L: image depicts P2Y₄-IR nerve bundle (J, red) also stained for PGP 9.5 (K, green). L: image depicts a representative merged image illustrating colocalization of P2Y₄ and PGP-9.5-IR in nerve bundles within the bladder smooth muscle. Scale bar = 50 μ m.

(23, 38). The prominence of P2X₂ receptors in the cat bladder and the absence of P2Y receptors, which have been implicated in inhibition of smooth muscle contraction in the rat and guinea pig bladder, suggest that purinergic transmission might be different in cats and rodents.

The function of ionotropic purinergic receptors in excitable tissues such as smooth muscle is easily explained; however, the contribution of these receptors to the activities of nonexcitable

tissue such as the urothelium is less clear. The presence of multiple types of P2X and P2Y receptors might be related to the multiple functions of the urothelium such as the maintenance of a barrier at the luminal surface of the bladder, participation in sensory transduction mechanisms, and antigen presentation in host defense mechanisms. Previous studies (1, 15, 33) revealed that chemical and mechanical stimuli can release ATP from urothelial cells and that exogenous ATP can

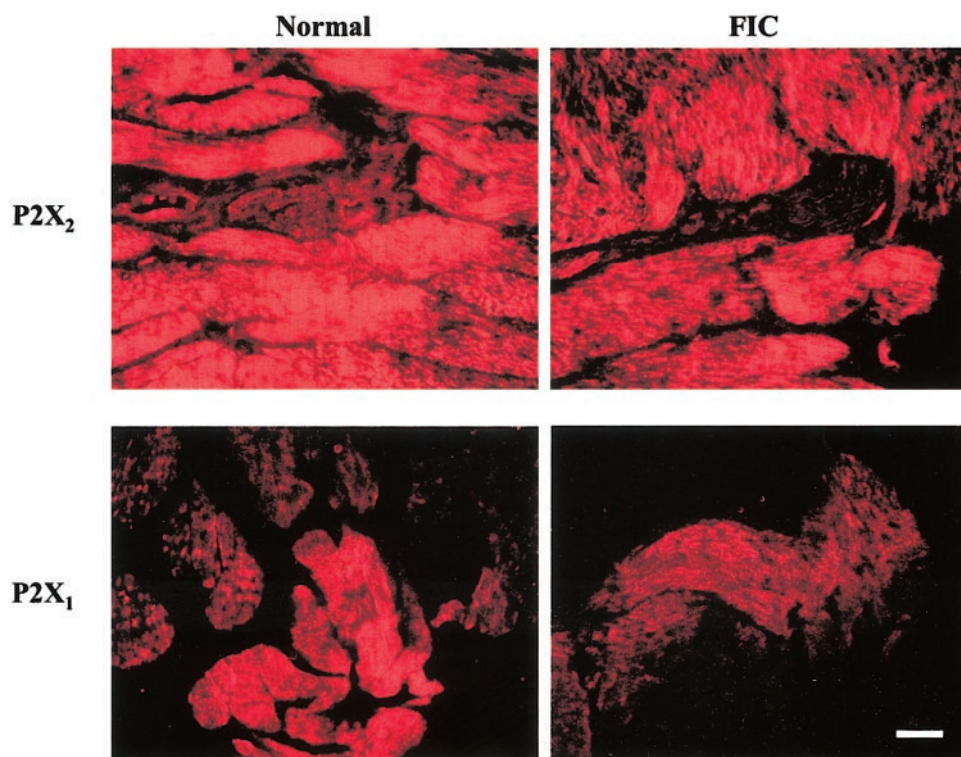


Fig. 5. Localization of P2X₁ and P2X₂ receptor IR in normal (left) and FIC (right) cat urinary bladder smooth muscle. Top: P2X₂-IR. Bottom: P2X₁-IR. Scale bar = 50 μ m. Note decrease in P2X₁ expression in FIC urinary bladder smooth muscle compared with smooth muscle from normal cat urinary bladder.

produce an increase in intracellular Ca^{2+} in urothelial cells, which might trigger the release of other transmitters (e.g., nitric oxide) from the cells. Mechanical stretch of urothelial cells elicits membrane trafficking from cytoplasmic vesicles to the plasma membrane to increase cell-surface area and cell size (37). This allows the urothelium to expand during bladder filling. This expansion of urothelial cell-surface area is modulated by protein kinases. Activation of P2Y (or P2X) receptors by locally released ATP might be involved in modulating this adaptive mechanism similar to the role of ATP in the regulation of cell volume (19).

In the cat urinary bladder, we identified three P2Y purinergic receptor subtypes (P2Y₁, P2Y₂, and P2Y₄) within the normal urothelium. There is evidence that P2Y receptors (in particular P2Y₂) are abundantly localized to the luminal surface of many types of polarized epithelial cells (12, 29). In fact, under physiological conditions, release of nucleotides in tissues by stimuli such as cough or osmotic stress can activate P2Y₂ receptors and this effect is terminated when the nucleotides are hydrolyzed by ectoenzymes. In the urothelium of the cat urinary bladder, P2Y₂ receptors are expressed both basolaterally as well as apically. P2Y receptors expressed basolaterally may be activated by nucleotides released into the blood in addition to release by nerves or other cell types.

P2X₃ and P2Y₄ receptors were detected in bladder nerves near the urothelium and in the smooth muscle. The receptors were colocalized with PGP9.5, indicating their neural localization. The receptors could be expressed in a heterogeneous population of nerves consisting of both afferent and efferent fibers both of which might be modulated by purinergic mechanisms. The P2X₃ receptor has been identified in bladder afferent nerves in a number of species including rat and mouse (6). Interest in the function of this receptor subtype in the

control of bladder activity arose from studies of P2X₃ receptor knockout mice, which have a lower voiding frequency and a larger bladder capacity than controls, suggesting these receptors are important in maintaining sensory thresholds. It is thought that activation of P2X₃ receptors on subepithelial bladder nerve fibers, which are well positioned to respond to ATP released from the epithelial cells, elicits algisia and bladder hyperreflexia (7, 10).

P2X₃ receptors might also be localized on parasympathetic efferent nerve terminals in the smooth muscle layers of the bladder. Exogenous ATP and ADP excite parasympathetic ganglion cells in the cat bladder (11, 35). An analysis of the structure-activity relationships among a series of purinergic agonists revealed a similarity between the neuronal excitatory effect and the excitatory effect of the agonists on the bladder smooth muscle, an action mediated by P2X receptors. Thus it seems reasonable to conclude that bladder parasympathetic neurons and possibly parasympathetic postganglionic nerves express P2X excitatory receptors. P2X₂ and P2X₄ receptors have also been identified in pelvic parasympathetic ganglia of the rat (40). Activation of these receptors induces excitatory effects mediated by membrane depolarization.

The role of P2Y₄ receptors in bladder nerves is uncertain. Although it remains to be established whether these nerves are afferent, activation of P2Y receptors increases $[\text{Ca}^{2+}]_i$ by releasing Ca^{2+} from intracellular stores, and in turn, alters the release of neuropeptides (31). Whether a similar mechanism exists in cat bladder neurons warrants further study.

What might be the significance of altered purinergic signaling and/or purinergic receptor subtype expression in FIC? In patients with idiopathic detrusor instability as well as IC (in human and cat), there is evidence that bladder purinergic system is augmented (20, 23, 24). For example, increased

release of ATP could activate bladder nerves (P2X₃) adjacent to the urothelium, leading to facilitation of bladder reflexes. In addition, ATP may also activate purinergic metabotropic receptors within the urothelium. In sensory neurons, ATP activation of metabotropic receptors has been demonstrated to enhance the sensitivity of other receptors (TRPV1) to chemical stimuli or temperature in a PKC-dependent manner (36). There is also evidence that kinins and neuropeptides such as substance P (SP) may potentiate purinergic-induced responses in the urinary bladder (27). Both SP and bradykinin have been demonstrated to potentiate purinergic-evoked currents in neurons expressing recombinant P2X₃ and P2X_{2/3} channels (28). Increased expression of SP has been demonstrated in bladder afferent neurons and in urinary bladder in models of cystitis as well as in both patients and animals diagnosed with IC (4, 26). Taken together, these data suggest urothelial cells may be part of a paracrine/autocrine purinergic signaling pathway that could regulate epithelial-afferent function and contribute to urothelial hypersensitivity and the sensitization of bladder sensory mechanisms in IC.

Our study also revealed a decrease in P2X₁ expression within the urothelium. It has been shown in other cell types that prolonged stimulation may result in changes in the surface localization of P2X receptors. In a number of tissues including smooth muscle, the P2X₁ receptor subtype has been demonstrated to be rapidly internalized following stimulation (13). That P2X₁ receptors are downgraded in FIC cat urothelium (which also displays increased ATP release under physiological conditions) suggests that a similar mechanism may also be present here.

At physiological concentrations, ATP has been demonstrated in a number of cell types to elicit the propagation of calcium "waves," via activation of P2Y receptors and mobilization of intracellular calcium. In the urinary bladder, physiological bladder distension releases ATP from urothelial cells, which could result in activation of P2Y receptors and mobilization of intracellular calcium. This increase in intracellular calcium may regulate a number of signaling pathways responsible for such downstream events as wound repair, cell migration, differentiation, and proliferation (8). It has been demonstrated that animals with FIC display a failure of the urothelial permeability barrier in addition to regions of damage to the urothelium. Thus abnormalities in P2Y receptor expression in addition to release of ATP in FIC may explain some of these changes in barrier function and ultrastructure.

Recent studies also revealed abnormalities in purinergic transmission as well as purinergic P2 receptor subtype expression in unstable bladder syndromes (20, 23, 24). How might these changes contribute to changes in bladder function observed in overactive bladder or interstitial cystitis? IC in both cats and humans induces both frequency and urgency. In patients with IC as well as those exhibiting detrusor instability and neurogenic bladders, there is a significant increase in magnitude of atropine-resistant bladder contractions compared with normal (24, 25). There is abundant evidence in rats and humans that ATP, acting on P2X purinergic receptors in bladder smooth muscle, may be involved in mediating these atropine-resistant NANC (nonadrenergic, noncholinergic)-type detrusor contractions (8). In both normal rat and human bladder smooth muscle, the P2X₁ purinergic receptor subtype predominates while the present study revealed both P2X₁ and

P2X₂ in cat bladder muscle. Although in humans nerve-evoked bladder contractions are nearly abolished by muscarinic antagonists, in disease states such as IC, there is a residual P2X-mediated component that is resistant to muscarinic blockade (24).

In conclusion, these studies demonstrated the presence of both P2X and P2Y receptors in bladder urothelium and bladder nerves in close proximity to the urothelium, with smooth muscle expressing mainly P2X receptors. Our findings also revealed that expression of P2 receptor subtypes is altered in FIC. Taken together, these results suggest that changes in purinergic transmission may contribute to symptoms in FIC.

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