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$P2X_3$ Knock-Out Mice Reveal a Major Sensory Role for Urothelially Released ATP

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The present study explores the possible involvement of a purinergic mechanism in mechanosensory transduction in the bladder using $P2X_3$ receptor knock-out ($P2X_3^{-/-}$) and wild-type control ($P2X_3^{+/+}$) mice. Immunohistochemistry revealed abundant nerve fibers in a suburothelial plexus in the mouse bladder that are immunoreactive to anti-$P2X_3$. $P2X_3$-positive staining was completely absent in the subepithelial plexus of the $P2X_3^{-/-}$ mice, whereas staining for calcitonin gene-related peptide and vanilloid receptor 1 receptors remained. Using a novel superfused mouse bladder–pelvic nerve preparation, we detected a release of ATP proportional to the extent of bladder distension in both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice, although $P2X_3^{-/-}$ bladder had an increased capacity compared with that of the $P2X_3^{+/+}$ bladder. The activity of multifiber pelvic nerve afferents increased progressively during gradual bladder distension (at a rate of 0.1 ml/min). However, the bladder afferents from $P2X_3^{-/-}$ mice showed an attenuated response to bladder distension. Mouse bladder afferents of $P2X_3^{+/+}$, but not $P2X_3^{-/-}$, were rapidly activated by intravesical injections of P2X agonists (ATP or α,β-methylene ATP) and subsequently showed an augmented response to bladder distension. By contrast, P2X antagonists [2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP and pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid] and capsaicin attenuated distension-induced discharges in bladder afferents. These data strongly suggest a major sensory role for urothelially released ATP acting via $P2X_3$ receptors on a subpopulation of pelvic afferent fibers.

Key words: ATP; $P2X_3$ receptor; pelvic afferents; immunohistochemistry; capsaicin; knock-out; mouse; urinary bladder

The storage and periodic evacuation of urine by the bladder is regulated by a complex neural control system that consists of the CNS and the afferent and efferent spinal nerves. It has been proposed that lumbosacral afferent fibers (pelvic afferents) of the urinary bladder principally regulate continence and micturition (Kuru, 1965; de Groat and Steers, 1990). These are small myelinated (A_δ) and unmyelinated (C) fibers that convey impulses from bladder wall receptors. Many investigators believe that myelinated sacral afferent fibers are primarily involved in the nonpainful micturition reflex (Bahns et al., 1986; Mallory et al., 1989), whereas unmyelinated sacral afferents are activated under painful, pathological conditions (Häbler et al., 1990). However, the underlying signal transduction mechanism for these afferents is poorly understood.

There is increasing interest in the role of locally released ATP in the bladder. Extracellular ATP has been reported to mediate excitation of sensory neurons via P2X receptors, which are ligand-gated ion channels. So far, seven P2X subunits ($P2X_{1–7}$) have been cloned, and six of them have been identified on sensory neurons (Collo et al., 1996; Ralevic and Burnstock, 1998). Of particular interest is the selective expression of $P2X_3$ subunits on small-diameter sensory neurons (Chen et al., 1995; Bradbury et al., 1998) and activation by P2X agonists of afferent fibers from a variety of tissues (Bland-Ward and Humphrey, 1997; Cook et al., 1997; Dowd et al., 1998; Hamilton et al., 2000; Rong et al., 2000). Burnstock (1999) recently put forward a hypothesis about purinergic mechanosensory transduction that proposed that in hollow organs, including the ureter and bladder, distension causes release of ATP from epithelial cells lining these organs and that ATP can then activate $P2X_3$ receptors on subepithelial sensory nerve terminals to evoke neural discharge. This is consistent with the studies of Ferguson et al. (1997), who demonstrated release of ATP from the serosal epithelium of rabbit bladder in response to stretch, as well as those of Namasivayam et al. (1999) who showed using an *in vitro* rat bladder preparation that prolonged exposure to a desensitizing dose of α,β-methylene ATP (α,β-meATP) significantly reduced the activity of mechanosensitive pelvic nerve afferents in response to distension. More recently, Cockayne et al. (2000) demonstrated that mice lacking the $P2X_3$ receptor gene ($P2X_3^{-/-}$) had marked urinary bladder hyporeflexia with reduced voiding frequency and increased voiding volume, raising the possibility that $P2X_3$ receptors are closely involved in mechanosensory transduction underlying the activation of afferent fibers during bladder filling.

In the present study, we have further explored the sensory role of $P2X_3$ receptors in the urinary bladder using $P2X_3$ receptor knock-out ($P2X_3^{-/-}$) and matching wild-type ($P2X_3^{+/+}$) mice. We aimed to (1) localize $P2X_3$ immunoreactivity in mouse urinary bladder; (2) quantify the release of ATP during graded distensions of the mouse bladder; and (3) establish an *in vitro* mouse bladder–pelvic nerve preparation and characterize the sensory nerve response to bladder distension and purinergic agonists.

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Preliminary results have been reported previously in abstract form (Vlaskovska et al., 2000).

MATERIALS AND METHODS

Animals

Four- to six-month-old male $P2X_3^{-/-}$ (27.8 ± 0.4 gm) and matching $P2X_3^{+/+}$ (30.8 ± 1.1 gm) mice were supplied by Roche Bioscience (Palo Alto, CA). Details about the generation of these mice have been described by Cockayne et al. (2000). The animals were kept and handled according to the regulations of the United Kingdom for the use of transgenic animals. The mice were killed by exposure to rising concentrations of CO_2 gas.

Immunohistochemistry

$P2X_3$ and Protein Gene Product 9.5 double labeling. The urinary bladder was dissected, and the trigone zone of the bladder was embedded in OCT compound (BDH/Merck, Leicester, UK) and frozen in isopentane precooled in liquid nitrogen. The tissues were sectioned at 12 μm using a Reichert Jung CM1800 cryostat, collected on gelatin-coated slides, and air-dried at room temperature. The slides were stored at −20°C and allowed to return to room temperature for at least 10 min before use. The sections were immersion-fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 min. Endogenous peroxidase was blocked by 10 min incubation in 50% methanol containing 0.4% hydrogen peroxide. Nonspecific protein binding sites were blocked by a 20 min incubation with 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate (Sigma, Poole, UK). Sections were incubated at room temperature overnight with $P2X_3$ antibody (diluted to 1 μg/ml with 10% NHS). The antibody was raised in New Zealand rabbits against a synthetic peptide corresponding to the C terminus of the cloned rat $P2X_3$ receptors (amino acid fragment 383–397; Oglesby et al., 1999). After this incubation all washes were performed using PBS containing 0.05% Tween 20 (Sigma). The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, Luton, UK) used at 1:500 for 1 hr, followed by incubation with ExtrAvidin peroxidase (Sigma, Dorset, UK) diluted 1:1500 for 30 min. The tyramide signal amplification kit (NEN Life Science Products, Boston, MA) was applied for 8 min, followed by incubation with Streptavidin fluorescein (Amersham Pharmacia Biotech, Bucks, UK) diluted 1:100 for 10 min. Slices were further incubated overnight with the axon marker Protein Gene Product 9.5 (PGP 9.5; UltraClone Ltd., Rossiters Farmhouse Wellow, UK) diluted 1:1000 in PBS containing bovine serum albumin (BSA), lysine, Triton X-100, and sodium azide. The secondary antibody, donkey anti-rabbit Cy3 (Jackson ImmunoResearch) was used at a dilution of 1:400 for 1 hr. The slices were observed under a Zeiss Axioplan microscope (Jena, Germany) at an excitation of 520 nm (for $P2X_3$ staining) and 570 nm (for PGP 9.5), and images were captured by a digital camera (Leica, Germany).

Confocal microscopy. The urothelium of the isolated urinary bladder was carefully stripped off from the smooth muscles and stretched as a whole-mount preparation with inner surface upward on a Sylgard plate. The tissue was fixed in 4% formaldehyde at room temperature for 1 hr and then incubated with 10% NHS to block nonspecific binding. Each preparation was incubated overnight with one of the following antibodies: anti-$P2X_3$ (5 μg/ml), anti-vanilloid receptor 1 (VR1) (1:2000; Chemicon, Temecula, CA), and anti-CGRP, all diluted in PBS containing BSA, lysine, Triton X-100, and sodium azide (all from Sigma). The secondary antibody, biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:500 in 1% NHS was applied for 1 hr followed by 1 hr incubation with streptavidin FITC (Amersham Pharmacia Biotech) diluted 1:100. The tissue was then incubated in Pontamine Sky Blue for 5 min to reduce autofluorescence. The tissue was observed under a confocal microscope at an excitation of 488 nm, and images were captured by a digital camera.

In vitro *urinary tract preparation and measurement of ATP release*

The whole urinary tract attached to the lower vertebrae and surrounding tissues was quickly isolated *en bloc* and placed in a chamber where it was continuously superfused with oxygenated (5% CO_2 and 95% O_2) Krebs' solution (contents in mM: NaCl 120; KCl 5.9; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 15.4; $CaCl_2$ 2.5; and glucose 11.5). The chamber temperature was kept at ~26°C. A 25 gauge needle was inserted into the lumen of the urinary bladder and was connected to a syringe-type infusion pump (sp210iw; World Precision Instruments, Sarasota, FL), a pressure transducer (NL108T2; Digitimer, Hertfordshire, UK), and a 100 μl Hamilton syringe via an Omnifit. This enabled infusion and withdrawal of medium (Krebs' solutions) at a constant rate (0.1 ml/min), recording of intraluminal pressure, and intravesical injection of drugs. The preparation was allowed to stabilize for at least 60 min.

ATP released from urothelial cells was measured in 100 μl samples taken from the intravesical medium. Before each distension, four or five samples were collected from wash-out medium at 5 min intervals (baseline ATP levels). The bladder was distended to intraluminal pressures of 7.5, 15, 20, and 25 mmHg for 2 min at random. ATP levels were quantified (in picomoles per milliliter) as previously described (Bodin and Burnstock, 1996). Briefly 50 μl aliquots were taken from each sample and pipetted in duplicates onto a multi-well non-phosphorescent microplate. This was placed in a luminometer (Lucyl; Anthos Labtec, Salzburg, Austria) and processed automatically by injection of 100 μl luciferin–luciferase reagent (Bio-Orbit, Turku, Finland) into each well. The light emission was counted for 10 sec, and ATP concentration was extrapolated from a calibration curve constructed using standard ATP solutions prepared in Krebs' solution (pH adjusted to 6 by 0.1 N HCl). The results are presented as mean ± SEM. Data were compared by Student's *t* test, and differences considered statistically significant at $p < 0.05$.

Electrophysiology

The mouse urinary tract was dissected and perfused as stated above. With the aid of a dissecting microscope, a branch of the pelvic nerve arising from the urinary bladder was dissected. The nerve activity was recorded with a suction glass electrode (tip diameter, 50–100 μm) connected to a Neurolog head stage (NL 100; Digitimer) and an AC amplifier (NL 104; Digitimer). Signals were amplified (10,000×), filtered (band-pass 200–4000 Hz), and relayed to a D310 spike processor (Digitimer) that discriminates neural impulses from noise with a manually set amplitude and polarity window. The nerve activity and the intraluminal pressure were recorded on tape and a computer with a power 1401 analog-to-digital interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). The nerve activity was counted and plotted as rate histogram.

In pilot studies, the characteristics of neuronal response to bladder distension and the stability of the model was tested by repeatedly filling the bladder with Krebs' solution at a rate of 0.1 ml/min and at intervals of 10–15 min. Consistent neuronal response to distension could be obtained for as long as 10 hr. To quantify the changes in nerve activity in response to bladder distension, each preparation was first distended at a rate of 0.1 ml/min until the nerve activity reached a plateau (control maximal activity), and this was repeated three times (interval between distension = 10 min). The average firing rate in every 15 sec period (i.e., for every 25 μl of injected volume) was counted, expressed as a percentage of the maximal activity (normalized nerve activity), and plotted against the volume of medium in the bladder. The effects of P2X agonists on the pelvic afferents and their response to distension were tested by injecting 0.1 ml solutions containing the agonists as a bolus into the empty bladder and distending the bladder 10 min thereafter. The effects of P2X antagonists and capsaicin were examined using a similar protocol.

Chemicals

ATP (disodium salt), α,β-meATP (lithium salt,), and pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS) (all obtained from Sigma, St. Louis, MO) were diluted to final concentrations in Krebs' solution, and the pH was adjusted to 7.4. Capsaicin (Tocris Cookson, Bristol, UK) was dissolved in DMSO as a 10 mM stock solution and diluted to final concentrations in Krebs' solution before use. All inorganic salts were purchased from BDH Laboratory Supplies (Poole, UK).

RESULTS

Immunohistochemical characterization of bladder sensory afferents

As illustrated in Figure 1, *A* and *D*, intense immunofluorescent staining for $P2X_3$ subunits was found in nerve bundles between the smooth muscle layer of the bladder wall in $P2X_3^{+/+}$, but not in $P2X_3^{-/-}$ mice. However, staining for PGP 9.5, an axon marker, was found in the bladder from both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice (Fig. 1*B,E*). The staining for $P2X_3$ subunits coex-

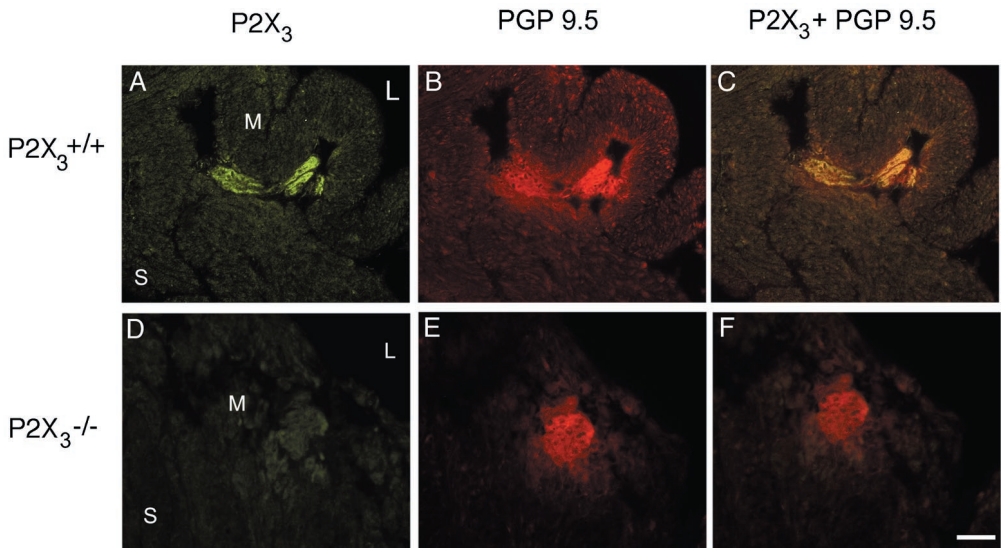

Figure 1. Double staining for $P2X_3$ and PGP 9.5 in the mouse urinary bladder. *Top* and *bottom panels* are sections of the bladder (with all layers of the bladder wall) from $P2X_3$ wild-type ($P2X_3^{+/+}$) and $P2X_3$ knock-out mice ($P2X_3^{-/-}$), respectively. *A*, *D*, Immunofluorescence (*green*) for $P2X_3$ receptor. Note that the nerve bundle within the smooth muscle coat is densely stained for the $P2X_3$ subunit. *B, E*, immunofluorescence (*red*) for PGP 9.5, an axon marker. *C*, *F*, Co-staining for $P2X_3$ and PGP 9.5. *L,* Luminal side of the bladder wall; *S*, serosal side of the bladder wall; *M*, smooth muscle. Scale bar, 100 μm.

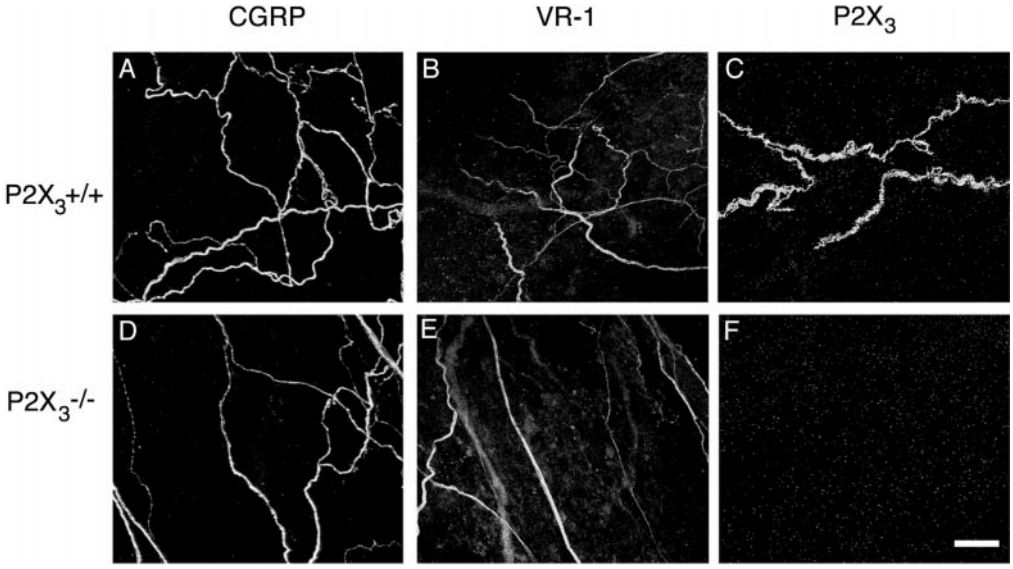

Figure 2. Immunostaining of the subepithelial sensory nerve plexus in the mouse bladder. Each panel represents the inner surface of a small piece of urothelium (clear of smooth muscles). *Top* and *bottom panels* are urothelium from $P2X_3$ wild-type ($P2X_3^{+/+}$) and $P2X_3$ knock-out mice ($P2X_3^{-/-}$), respectively. *A*, *D*, Immunofluorescence for CGRP; *B, E*, Immunofluorescence for VR1; *C, F*, Immunofluorescence for $P2X_3$ subunits. Scale bar, 100 μm.

isted with that of PGP 9.5 in the $P2X_3^{+/+}$, but not in the $P2X_3^{-/-}$ mouse bladder (Fig. 1*C,F*).

Whole-mount bladder urothelial preparations examined with confocal microscopy revealed a dense suburothelial plexus of CGRP-immunoreactive nerve fibers in $P2X_3^{+/+}$ as well as $P2X_3^{-/-}$ mice (Fig. 2*A,D*). Similarly, VR1-immunoreactive fibers (Fig. 2*B,E*) were also found in the suburothelial plexus from both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. Although numerous $P2X_3$-positive fibers were found in the suburothelial plexus and in those projections between urothelial cells in the bladder of $P2X_3^{+/+}$ mice (Fig. 2*C*), none were seen in the suburothelial plexus of $P2X_3^{-/-}$ mice (Fig. 2*F*).

Distension-induced ATP release from the urothelium

The bladder was distended by intravesical infusion of Krebs' solution at a constant rate (0.1 ml/min) until the intraluminal pressure reached a certain level. It was noticed that the bladders of $P2X_3^{-/-}$ mice typically had a larger capacity than those of $P2X_3^{+/+}$ mice such that the same levels of intravesical pressure could be achieved after infusion of larger volumes of medium. In bladders from $P2X_3^{+/+}$ mice, 7.5 mmHg was achieved with 110 ± 5 μl medium, 15 mmHg with 215 ± 10 μl, 20 mmHg with 320 ± 20 μl, and 25 mmHg with 430 ± 15 μl. In bladders from $P2X_3^{-/-}$ mice, the same intravesical pressure was produced by infusion of 135 ± 15, 245 ± 15, 360 ± 20, and 480 ± 25 μl, respectively. The basal release of ATP was measured in the samples taken from the medium during every predistension period. The baseline levels of ATP were similarly low during all predistension periods in both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice, being 0.5 ± 0.1 and 0.6 ± 0.1 pmol/ml, respectively. The threshold for distension-evoked ATP release was 3.5–4.0 mmHg, which corresponded to intravesical volume of 60–70 μl for the $P2X_3^{+/+}$ mouse bladder. With increasing intravesical volume (and pressure), ATP level increased progressively. Thus, distension to an intravesical pressure of 7.5 mmHg produced a several-fold incremental increase of ATP over the baseline level. The plateau of intravesical ATP levels was reached at an intravesical pressure of 20 mmHg, which corresponded to intravesical volume of 300 ± 20 μl. Distension of $P2X_3^{-/-}$ bladder produced an identical pattern of ATP release. The results of this series of experiments are summarized in Table 1.

Table 1. Distension-induced release of ATP in $P2X_3$ wild-type and $P2X_3$ knock-out mouse urinary bladder

Intravesical pressure	Release of ATP (pmol/ml)	
	$P2X_3$ wild-type	$P2X_3$ knock-out
Basal pressure	0.5 ± 0.1 (15)	0.6 ± 0.1 (4)
7.5 mmHg	4.1 ± 1.0** (15)	3.6 ± 1.8* (3)
15 mmHg	10.7 ± 1.9** (11)	11.6 ± 3.8* (4)
20 mmHg	20.8 ± 3.2** (10)	21.9 ± 6.6* (3)
25 mmHg	20.9 ± 3.2** (4)	21.9 ± 3.2* (4)

*$p < 0.05$ and **$p < 0.001$ versus basal release (Student's t test). In parentheses are the number of animals.

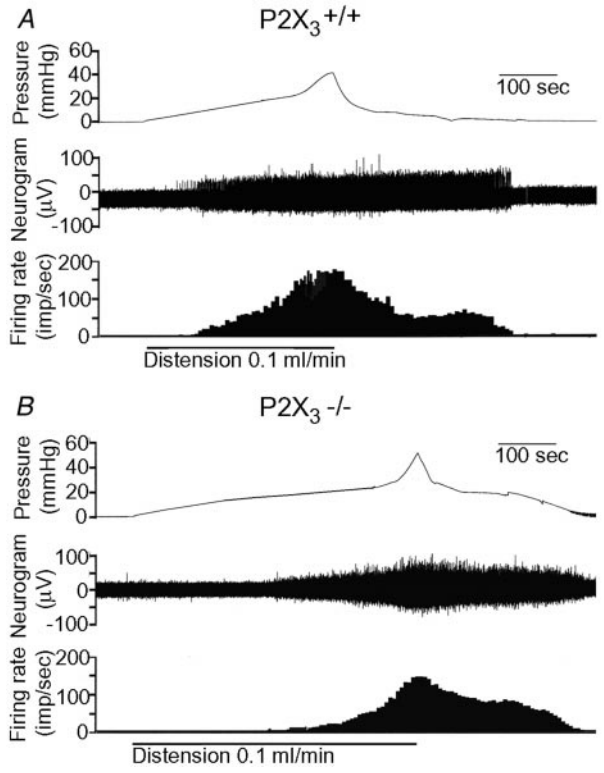

Figure 3. Distension-induced discharges in mouse bladder afferents. *A*, Wild-type mice ($P2X_3^{+/+}$). *B*, $P2X_3$ knock-out mice ($P2X_3^{-/-}$). The bladder was distended at a constant rate of 0.1 ml/min. Note the much delayed activation of the bladder afferents in the $P2X_3^{-/-}$ preparation.

Activity of pelvic afferent fibers evoked by distension of the bladder and P2X agonists

The pelvic afferent fibers from the urinary bladder of $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice both had low background activity (0–10 spikes/sec). Examples of distension-induced changes in intravesical pressure and discharges in pelvic afferents in bladder–pelvic nerve preparations from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice are illustrated in Figure 3. Figure 4 summarizes the data from this series of experiments. In most preparations, biphasic activation of afferent fibers in association with increases in intravesical pressure was observed with an initial slow rise followed by a later rapid increase in pressure and nerve activity. However, the pelvic afferents of $P2X_3^{-/-}$ mice were less sensitive to bladder distension than those of $P2X_3^{+/+}$ mice (Fig. 3, compare *A*, *B*). In the $P2X_3^{+/+}$ mice, nerve activity started to increase when the bladder was distended to a volume of 115.6 ± 11.6 μl (intravesical pressure of 5.7 ± 1.0 mmHg; $n = 8$), whereas in the $P2X_3^{-/-}$ mice, the threshold for significant increase in nerve activity was 276.7 ± 24.0 μl (intravesical pressure of 10.2 ± 1.6 mmHg; $n = 6$) (Fig. 4). In $P2X_3^{+/+}$ mice, nerve activity reached a peak when the bladder was distended to a volume of 472.6 ± 51.8 μl (intravesical pressure of 36.1 ± 4.7 mmHg; $n = 8$), whereas in the $P2X_3^{-/-}$ mice, peak nerve activity was achieved at significantly larger intravesical volumes (729.1 ± 57.3 μl, corresponding to a pressure of 40.5 ± 6.1 mmHg; $n = 6$) (Fig. 4).

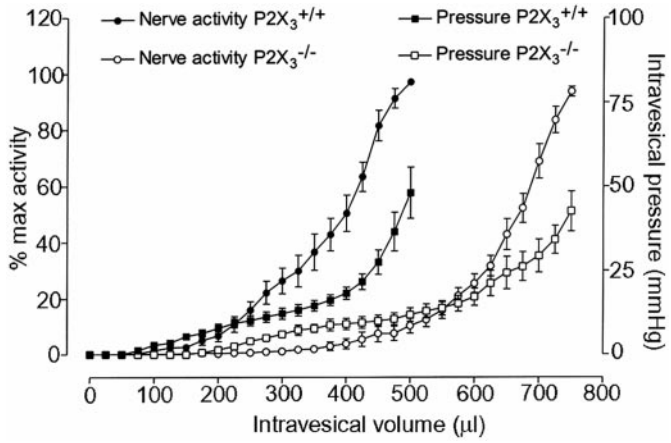

Figure 4. A summary of the alterations in intravesical pressure and bladder afferent activity during distension of the bladder. The nerve activity was normalized and expressed as the percentage of maximal activity. Data representative of an $n = 8$ for $P2X_3$-wild type mice ($P2X_3^{+/+}$) and an $n = 6$ for $P2X_3$-knock-out mice ($P2X_3^{-/-}$).

The effects of P2X agonists (α,β-meATP and ATP) and capsaicin on the pelvic afferents of $P2X_3^{+/+}$ mice ($n = 7$) and $P2X_3^{-/-}$ mice ($n = 5$) are shown in Figure 5. The agonists were injected into the empty bladder as a 100 μl bolus. Injecting 100 μl of vehicle into the bladder did not evoke significant changes in intravesical pressure and nerve activity. In the $P2X_3^{+/+}$ mice (Fig. 5*A1*), α,β-meATP (100–300 μM) induced a rapid increase in the discharge of the pelvic afferents, and the response lasted for 5–10 min. Similarly, ATP (Fig. 5*B1*) induced a rapid excitation of the pelvic afferents, but the minimal concentration of ATP (1 mM) required to evoke a significant neural response was ~10-fold higher than that of α,β-meATP (100 μM). Neither ATP nor α,β-meATP had any significant effect on the activity of the pelvic nerve of the $P2X_3^{-/-}$ mice (Fig. 5*A2,B2*). However, capsaicin (30 μM) induced significant afferent activation in both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ preparations. All these agonists also produced similar small increases in intravesical pressure in both $P2X_3^{+/+}$ (Fig. 5*C1*) and $P2X_3^{-/-}$ (Fig. 5*C2*) preparations.

The effects of α,β-meATP and capsaicin agonists on the afferent nerve response to distension were further examined by distending the bladder 10 min after the administration of the agonists. As demonstrated in Figures 6 and 7, α,β-meATP did not affect the pressure changes during distension. However, in $P2X_3^{+/+}$ preparations the afferent nerve response to distension was greatly potentiated by preinfusion of agonist, in that nerve activity increased faster and reached a greater peak level than that observed with vehicle alone (Figs. 6, 7). Recovery of the distension-induced afferent nerve responses was achieved after washout of the agonist. The potentiation was not seen in the $P2X_3^{-/-}$ preparation ($n = 2$). In contrast, after administration of capsaicin, the afferent nerve response to bladder distension was

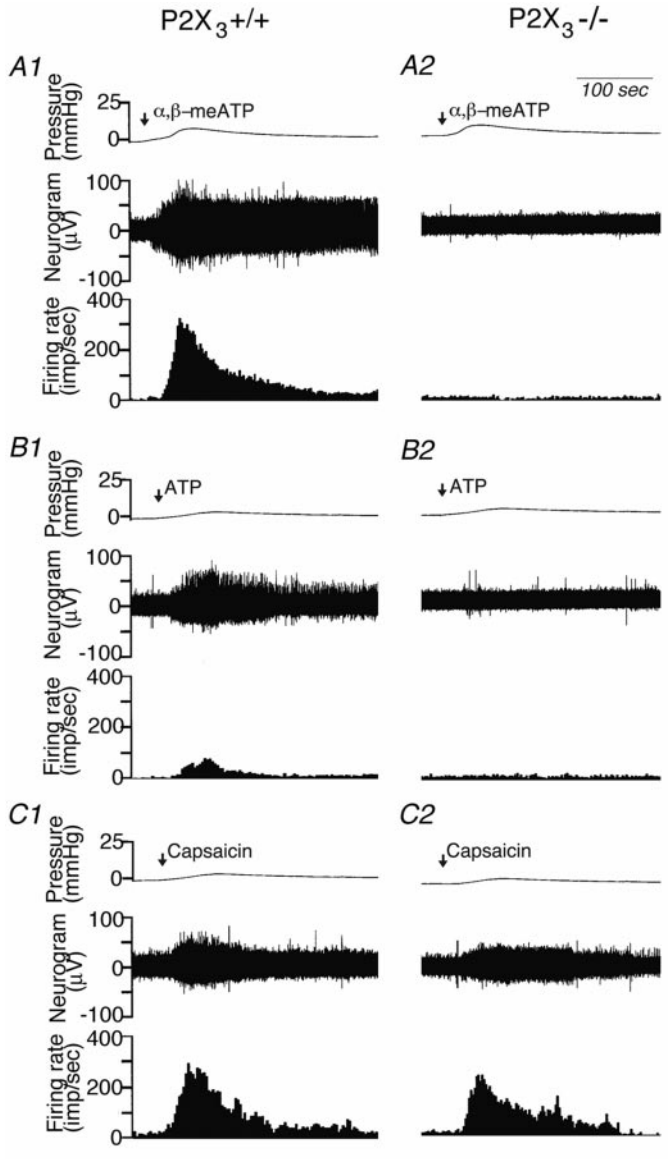

Figure 5. Responses of multifiber bladder afferents to intravesical administration of P2X agonists and capsaicin. *Left*, Wild-type control mice ($P2X_3^{+/+}$). *Right*, $P2X_3$ knock-out mice ($P2X_3^{-/-}$). Agonists (α,β-meATP, 100 μM; ATP, 1 mM; capsaicin, 30 μM) were injected into the empty bladder in a volume of 100 μl. All recordings on the *left column* are from same $P2X_3^{+/+}$ mouse, and those on the *right column* are from same $P2X_3^{-/-}$ mouse.

significantly attenuated with peak activity reduced to 34.7 ± 5.8% ($n = 5$; $p < 0.01$) of the control.

The neural response to P2X agonists in the $P2X_3^{+/+}$ control mice could be blocked by the P2X antagonists PPADS ($n = 4$) and 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) ($n = 4$). This is exemplified in Figure 8, which illustrates the attenuation in nerve activity induced by intravesical injection of α,β-meATP after, but not before, preincubating the preparation intravesically with TNP-ATP (Fig. 8, compare *A, B*). Both PPADS and TNP-ATP were also able to reduce the neural response to bladder distension with a representative recording shown in Figure 9. Distension-induced peak activity was reduced after pretreatment with TNP-ATP (30 μM) and PPADS (300 μM) to 65.3 ± 18.4% ($n = 4$; $p < 0.05$) and 47.1 ± 17.3% ($n = 4$; $p < 0.01$) of the vehicle control, respectively.

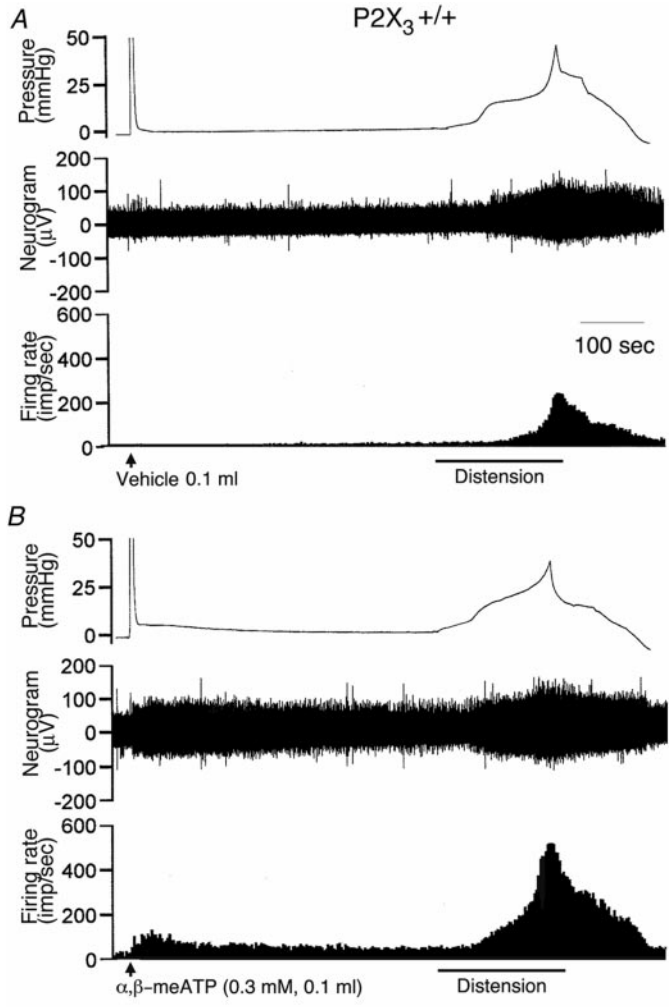

Figure 6. Effects of α,β-meATP on the distension-induced changes in multifiber bladder afferent activity in a $P2X_3$ wild-type ($P2X_3^{+/+}$) mouse. *A* and *B* are sequential recordings in same preparation. In *A*, an injection of vehicle (Krebs', 0.1 ml) was followed by bladder distension (0.1 ml/min). In *B*, distension (0.1 ml/min) was preceded by an injection of α,β-meATP (0.3 mM, 0.1 ml), which itself elicited afferent discharges and potentiated distension-induced discharges.

DISCUSSION

Morphology of the sensory innervation of the bladder

The major function of the urinary bladder is to store and evacuate urine. The significance of the sensory innervation of the bladder is to detect the filling of the bladder and convey this information to the CNS to evoke the micturition reflex. The innocuous sensory information about bladder fullness is transformed to a painful sensation (or discomfort) when the intravesical pressure reaches a threshold of 30–50 mmHg (Häbler et al., 1990). Two types of afferent fibers are involved, namely myelinated Aδ- and unmyelinated C-afferent fibers of the pelvic and the hypogastric nerves (McGuire, 1986), which comprise low- and high-threshold mechanosensory receptors (Sengupta and Gebhart, 1994). Detailed immunohistochemical (CGRP) and immunofluorescent (synaptophysin) studies have revealed dense plexuses of afferent axons in a close apposition to the urothelium (Gabella and Davis, 1998). The afferent axons were shown to be associated with four major targets: the base of the urothelium, the space between urothelial cells, blood capillaries, and smooth

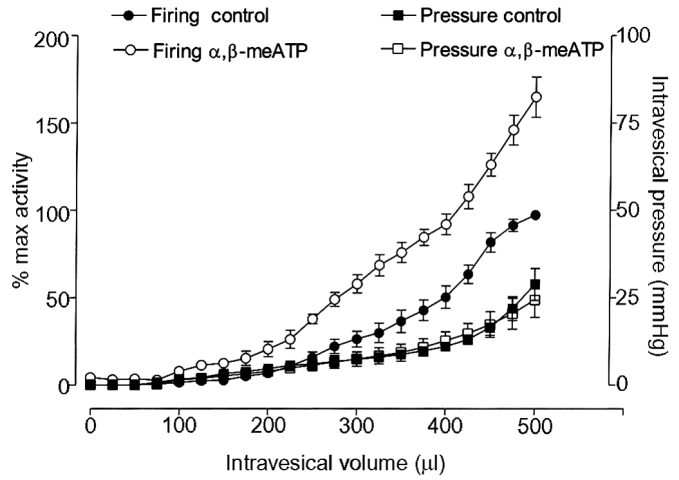

Figure 7. A summary of the effects of α,β-meATP on distension-induced changes in bladder afferent activity in $P2X_3$ wild-type ($P2X_3^{+/+}$) mice. The experimental protocol was the same as shown in Figure 6. The nerve activity was normalized and expressed as a percentage of the maximal firing rate reached during the control distension with vehicle. Data were pooled from six $P2X_3^{+/+}$ mouse bladder–nerve preparations.

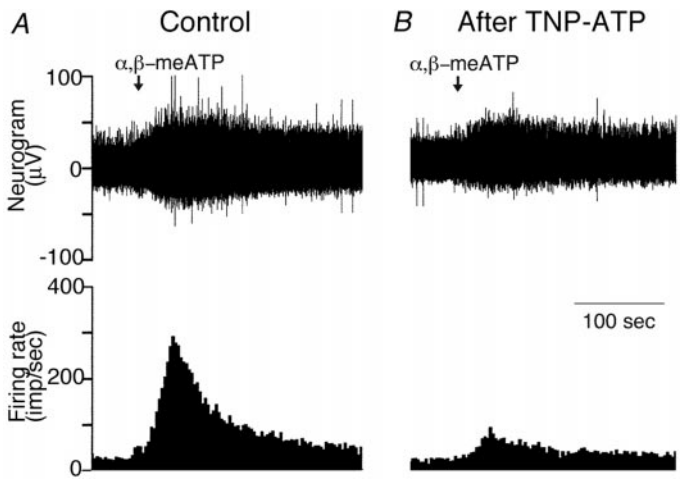

Figure 8. Effects of TNP-ATP on bladder afferent responses to α,β-meATP. *A*, An intravesical injection of α,β-meATP (100 μM, 0.1 ml) induced significant discharges in the bladder afferents. *B*, The preparation was incubated intravesically with the P2X antagonist TNP-ATP (30 μM, 0.1 ml) for 10 min; a subsequent injection of α,β-meATP (100 μM, 0.1 ml) after removal of the TNP-ATP produced little change in nerve activity.

muscle cells. In the mucosa, all afferent axons are located between urothelial cells or in the suburothelial plexus adjacent to the basal surface of urothelium. Immunohistochemical studies have revealed that although the detrusor muscle expresses $P2X_1$ receptors (Ferguson, 1999; Lee et al., 2000), $P2X_3$ receptors are present in the urothelium (Ferguson, 1999) and specifically on afferent nerve fibers (Cockayne et al., 2000). In agreement with these data, we found abundant CGRP, VR1, and $P2X_3$ receptor-immunoreactive nerve fibers running between urothelial cells, in the suburothelial plexus, and perivascularly in the urinary bladder of $P2X_3^{+/+}$ mouse (Fig. 2). $P2X_3$ immunostaining coexists with staining for the neuronal marker PGP 9.5 (Fig. 1), indicating the neuronal localization of $P2X_3$ receptors. As expected, $P2X_3$ receptor immunoreactivity was completely absent in the bladder of $P2X_3^{-/-}$ mice, but the immunoreactivity for CGRP and VR1

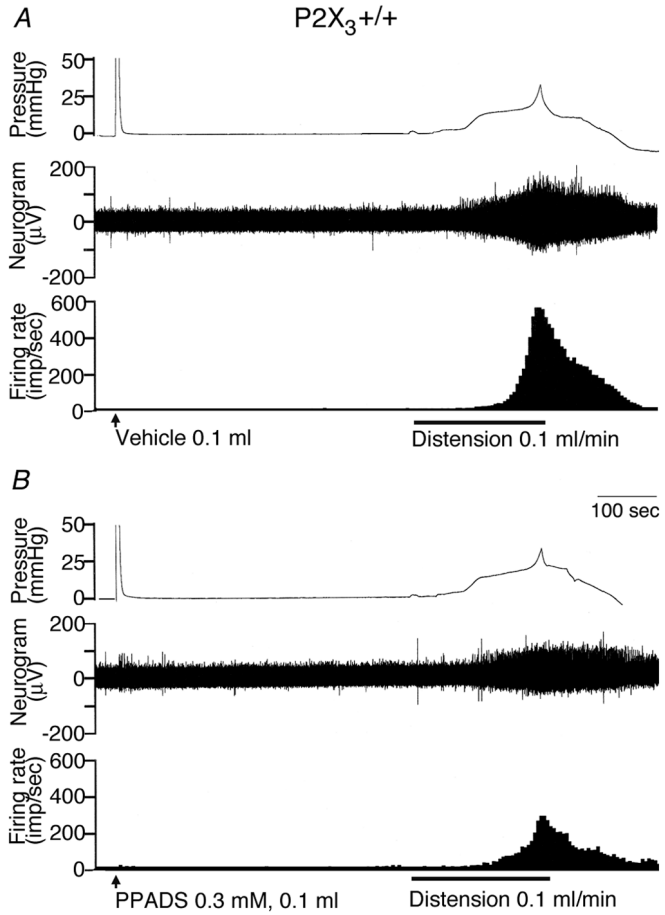

Figure 9. Effects of PPADS on the distension induced changes in bladder afferent activity in a $P2X_3$ wild-type ($P2X_3^{+/+}$) mouse bladder–nerve preparation. *A*, Nerve activity in control situation. *B*, Nerve activity after application of PPADS (300 μM).

seemed unaltered (Fig. 2). The importance of the $P2X_3$-positive afferents in sensory signal transduction in the bladder is signified by the findings of Cockayne et al. (2000) that $P2X_3$-deficient mice display marked urinary bladder hyporeflexia.

Distension induced release of ATP from the bladder

In a rabbit bladder preparation, Ferguson et al. (1997) recorded changes in short-circuit currents and transepithelial potential that were accompanied by a substantial release of ATP from the serosal, but not luminal side of the urothelium in response to stretch. It was suggested that urothelium was a sensor for pressure in the bladder, which released ATP through nonvesicular mechanisms. In the present study, we detected a significant release of ATP into the lumen during distension of the mouse urinary bladder. In normal control mice, the threshold for the release of ATP was 3.5–4.0 mmHg of intravesical pressure, indicating that innocuous filling of the bladder may evoke release of ATP. We further demonstrated that distension-induced release of ATP is preserved in $P2X_3^{-/-}$ mice.

Electrical activity of the pelvic nerve fibers from the bladder

The electrophysiological properties of sensory innervation in the lower urinary tract have been studied extensively in the rat (Mallory et al., 1989) and cat *in vivo* (Bahns et al., 1986; Janig and Morrison, 1986; Häbler et al., 1990) and more recently in the rat

in vitro (Namasivayam et al., 1998, 1999). The mouse has so far been a neglected species in the study of neural control of the urinary tract, and little is known about the functional properties of afferent nerves from the mouse bladder. However, with more transgenic mice becoming available, this species is becoming an increasingly important experimental animal in bridging the information from molecular biology and systems physiology. In this paper, we describe a novel *in vitro* mouse bladder–pelvic nerve preparation. Using this model, we have, for the first time, examined the characteristics of the pelvic afferent response to bladder distension. The response consists of a progressive increase in firing rate in association with rises in intravesical pressure. Our observations indicate that this preparation is a relatively simple and reliable model for investigating sensory signal transduction mechanisms in the urinary bladder.

Namasivayam et al. (1999) recently reported the use of an *in vitro* rat bladder–pelvic nerve preparation to demonstrate that distension induced by an intravesical infusion of a solution containing 10 μM α,β-meATP produced slightly greater responses at first and then significantly attenuated responses in pelvic afferents. These effects of α,β-meATP were attributed to the activation and subsequent desensitization of P2X receptors located on afferent terminals on the urinary bladder. In support of this concept, we have localized $P2X_3$ receptor immunoreactivity on afferent nerve fibers located in the mouse urinary bladder. Our electrophysiological observations demonstrate that intravesical applications of P2X agonists (ATP or α,β-meATP) induce a rapid excitation of bladder afferents in the $P2X_3{}^{+/+}$ mice. Furthermore, α,β-meATP greatly potentiated subsequent distension-induced afferent discharges. This seems to contradict the observations of Namasivayam et al. (1999). However, this discrepancy may be attributable to the difference in the concentration and the speed of delivery of the agonist. Although we injected α,β-meATP (100–300 μM, 0.1 ml) into the bladder as a bolus, they distended the bladder slowly with α,β-meATP (10 μM).

A significant change in electrophysiological properties of bladder afferents in $P2X_3{}^{-/-}$ mice is their diminished response to P2X agonists (Fig. 5) and their attenuated response to bladder distension, which was manifested by a significantly delayed and increased threshold of activation of the afferents when compared with those from $P2X_3{}^{+/+}$ mice (Figs. 3, 4). This strongly indicates that $P2X_3$ subunits on afferent terminals may play an important part in mechanosensory transduction in the urinary bladder. However, as illustrated in Figure 4, $P2X_3{}^{-/-}$ bladder generates less intravesical pressure for a given distension. On one hand, this is not unexpected if mechanosensory transduction in the bladder involves a purinergic component of $P2X_3$ subunits—a deficiency of which may lead to increased threshold for micturition and consequently compensatory increase in bladder capacity. On the other hand, it is likely that this reduced bladder pressure response (or increased bladder capacity) in the $P2X_3{}^{-/-}$ mice may also partially account for the attenuated nerve response to bladder distension.

Previous studies indicate that there may be different functional classes of bladder afferents. Aδ afferents in the cat respond in a graded manner to distension of the bladder (Janig and Morrison, 1986) and are activated by noxious stimuli as well. On the other hand, C fibers in the cat have very high mechanical thresholds and commonly do not respond to even high levels of intravesical pressure (Häbler et al., 1990). The activity in some of these afferents, however, is triggered by chemical irritation of the bladder mucosa (Häbler et al., 1990) or cold (Fall et al., 1990). Single-unit studies will be necessary to determine whether a purinergic mechanism preferentially modulates the sensory transduction in one of the different classes of bladder afferents.

In other organs, capsaicin has been used to differentiate between innocuous and nociceptive (Aδ and C) fibers. However, whereas afferents from the bladder consist largely of Aδ and C fibers, both are activated by capsaicin. In the present study capsaicin was able to significantly attenuate afferent response to distension and intravesical administration of P2X agonists. This is consistent with the immunohistochemical colocalization of $P2X_3$ and VR1 receptors in afferent nerve fibers. Interestingly, rats treated with capsaicin as neonates were found to excrete less urine (Holzer-Petsche and Lembeck, 1984). Their bladders were found to have a larger capacity (5 ml) than normal controls (1 ml), and systemic capsaicin treatment was able to block the rhythmic contraction of detrusor muscles in response to distension. Clinically, intravesical administration of capsaicin has been proven effective in treating incontinence (Chancellor and de Groat, 1999). Given that P2X agonists and capsaicin can affect the activity of the same set of afferent fibers, $P2X_3$ agonists/antagonists may be of therapeutic use in urological conditions arising from disorders affecting the sensory mechanisms in the bladder.

In summary, we have demonstrated the presence of $P2X_3$ receptors on afferent nerve fibers closely associated with the urothelium and the release of ATP by distension of the mouse urinary bladder in a novel *in vitro* mouse bladder–pelvic nerve preparation. We found that P2X agonists were able to activate pelvic afferents and potentiate their response to bladder distension in $P2X_3{}^{+/+}$, but not in $P2X_3{}^{-/-}$ mice that showed attenuated pelvic afferent response to bladder distension. These data strongly suggest a major sensory role of urothelially released ATP via activation of $P2X_3$ receptors on a subpopulation of pelvic afferent nerve fibers.

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